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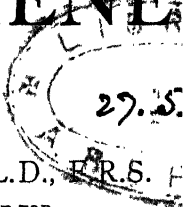
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# THE JOURNAL OF HYGIENE

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THE FERMENTATION OF SALTS OF ORGANIC  
ACIDS AS AN AID TO THE DIFFERENTIATION  
OF BACTERIAL TYPES.

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(With Plates I and II.)

IN the considerable amount of work already done on the decomposition of the salts of organic acids by bacteria, more attention has been directed to the products of metabolism than to the possibility of differentiating bacterial types by this means.

Koser (1923), in a recent paper on this subject, gives an exhaustive account of the previous work, and shows that no systematic attempt has been made hitherto to apply the results to the differentiation of bacteria.

In the present work it is not proposed to advocate the substitution of organic salts for "sugars" except in those cases in which "sugar reactions" are untrustworthy or fail altogether to differentiate certain serologically well-defined types of bacteria, *e.g.* those of the *Salmonella* group, but we think it reasonable to point out that there are two good reasons for such substitution: (1) the purity of the rarer "sugars" is often doubtful, and (2) the cost of such substances as *inositol* and *trehalose*, a diglucose recommended by Jordan (1923), limits, if it does not actually prohibit their use by investigators. Again, the value of aërogenesis, an important feature of sugar fermentation tests, has been impugned by many workers; Ledingham and Penfold (1915) remark that "many paratyphoid strains give little or no gas in the sugars they normally ferment," and Jordan (1923) states that gas formation in *inositol* media is very variable and therefore an unsafe basis for judgment.

*Inositol* was employed by Andrewes and Neave (1921) in differentiating the *B. paratyphosus* B and "Mutton" from other types, but they found that one out of twelve "B" types and one out of eight "Mutton" types failed to ferment this substance, and Jordan (1923) also records discrepancies.

It is unnecessary to cite further references here to variations in the fermentation of "sugars" by bacteria, but those interested in this aspect of the question may be referred to the work of Gurney-Dixon (1919) who quotes the important observations of Penfold, Arkwright and many other observers.



*Fermentation of organic salts; change in reaction of medium and aërogenesis.*

We first investigated the bacterial fermentation of certain organic salts mainly to determine to what extent these reactions could be employed in the differentiation of bacterial types. Salts of the following open-chain acids were used: the basic radicles are shown in brackets after the name and formula of the acid.

(1) *Monocarboxylic acids*

Formic acid  $\text{H}.\text{COOH}$ . (Na, K)  
 Acetic acid  $\text{CH}_3.\text{COOH}$ . (Na, K)  
*n*-Butyric acid  $\text{C}_3\text{H}_7.\text{COOH}$ . (Na)  
*n*-Valeric acid  $\text{C}_4\text{H}_9.\text{COOH}$ . (Na)  
*iso*-Valeric acid  $\text{C}_4\text{H}_9.\text{COOH}$ . (Na)

(2) *Dicarboxylic acids*

(a) Saturated:

Oxalic acid  $\text{COOH}.\text{COOH}$ . ( $\text{Na}_2$ )  
 Malonic acid  $\text{COOH}.\text{CH}_2.\text{COOH}$ . ( $\text{Na}_2$ )  
 Succinic acid  $\text{COOH}.\text{CH}_2.\text{CH}_2.\text{COOH}$ . ( $\text{Na}_2$ )  
 Glutaric acid  $\text{COOH}.\text{(CH}_2\text{)}_3.\text{COOH}$ . ( $\text{Na}_2$ )  
 Pimelic acid  $\text{COOH}.\text{(CH}_2\text{)}_5.\text{COOH}$ . ( $\text{Na}_2$ )

(b) Unsaturated:

Fumaric acid  $\text{COOH}.\text{CH}:\text{CH}.\text{COOH}$ . ( $\text{Na}_2$ )  
 Maleic acid  $\text{COOH}.\text{CH}:\text{CH}.\text{COOH}$ . ( $\text{Na}_2$ )

(3) *Tricarboxylic acid*

Aconitic acid  $\text{COOH}.\text{CH}:\text{C}(\text{COOH}).\text{CH}_2.\text{COOH}$ . ( $\text{Na}_2$ )

(4) *Hydroxymonocarboxylic acids*

Glycollic acid  $\text{CH}_2\text{OH}.\text{COOH}$ . (Na)  
 Lactic acid  $\text{CH}_3.\text{CHOH}.\text{COOH}$ . (Na)

(5) *Hydroxydicarboxylic acids*

Tartronic acid  $\text{COOH}.\text{CHOH}.\text{COOH}$ . ( $\text{Na}_2$ )  
 Malic acid  $\text{COOH}.\text{CH}_2.\text{CHOH}.\text{COOH}$ . ( $\text{Na}_2$ )  
*d*-Tartaric acid  $\text{COOH}.\text{CHOH}.\text{CHOH}.\text{COOH}$ . ( $\text{Na}_2$ , Na, K,  $\text{K}_2$ )  
*l*-Tartaric acid  $\text{COOH}.\text{CHOH}.\text{CHOH}.\text{COOH}$ . ( $\text{Na}_2$ )  
*dl*-Tartaric acid (racemic acid)  $\text{COOH}.\text{CHOH}.\text{CHOH}.\text{COOH}$ . ( $\text{Na}_2$ ,  $\text{K}_2$ )  
 Mesotartaric acid  $\text{COOH}.\text{CHOH}.\text{CHOH}.\text{COOH}$ . ( $\text{Na}_2$ )  
 Mucic acid  $\text{COOH}.\text{(CHOH)}_4.\text{COOH}$ . ( $\text{Na}_2$ )

(6) *Hydroxytricarboxylic acid*

Citric acid  $\text{CH}_2.\text{(COOH)}.\text{C}(\text{OH}).\text{(COOH)}.\text{CH}_2.\text{COOH}$ . ( $\text{Na}_2$ ,  $\text{K}_2$ )

(7) *Ketomonocarboxylic acid*

Laevulinic acid  $\text{CH}_3.\text{CO}.\text{CH}_2.\text{CH}_2.\text{COOH}$ . (Na)

These salts were used individually in a concentration of 1 per cent. in a basal medium of ordinary nutrient broth having a "reaction" of pH 7.4. Phenol red was employed as an indicator and Durham's tubes to record gas-formation. Sterilisation was accomplished by autoclaving at 115° C. for 20 minutes.

The behaviour of the following organisms on the "salt media" was studied.

4 strains of <i>B. typhosus</i>	1 strain of <i>B. psittacosis</i>
2 " <i>B. paratyphosus</i> A	2 strains of <i>B. coli communis</i>
3 " <i>B. paratyphosus</i> B	1 strain of <i>B. coli communior</i>
3 " <i>B. paratyphosus</i> C	2 strains of <i>B. acidilactici</i>
3 " <i>B. enteritidis</i> , Gärtner	1 strain of <i>B. aërogenes</i>
2 " <i>B. suispestifer</i>	1 " <i>B. cloacae</i>
2 " <i>B. aertrycke</i> , Mutton type	1 " <i>B. pneumoniae</i> , Friedländer
1 strain of <i>B. aertrycke</i> , Newport type	1 " <i>B. morgan</i> I
2 strains of <i>B. abortivo equinus</i>	1 " <i>V. cholerae</i>
1 strain of <i>B. pullorum</i>	1 " <i>B. gallinarum</i>

The media were sown from fresh well-grown broth cultures, the inoculating dose being a 3.0 mm. loopful of the culture. The tubes were then incubated at 37° C. and daily observations of aërogenesis and changes in reaction were made up to the end of 96 hours.

Of the salts tested very few were found to be readily acted upon by the organisms, and of these few the salts of formic, citric and *d*-tartaric acids were by far the most satisfactory. For the purposes of this paper it will suffice to state briefly the general behaviour of the various organisms on these three salts.

*Formates.* All the above organisms with the exception of *B. typhosus*, *B. gallinarum*, *V. cholerae* and one strain of *B. abortivo equinus*, formed an abundance of gas and produced a marked change to alkalinity in the medium after 24 hours at 37° C.

*Citrates.* Trisodium citrate gave very irregular results. A specially prepared chemically pure salt was used; with this *B. paratyphosus* A always showed inhibition of growth and never produced gas or caused any change in reaction. Most of the other organisms, except *B. pullorum*, produced a slight, or rarely a marked, degree of acidity followed after 48 hours by a moderate degree of alkalinity. Apparent gas-formation was unusual and no bacterial strain was found to yield gas constantly; *B. paratyphosus* B, *B. paratyphosus* C, *B. suispestifer*, *B. enteritidis*, Gärtner, *B. aertrycke*, Mutton, *B. aertrycke*, Newport, *B. abortivo equinus*, *B. aërogenes*, *B. cloacae*, *B. pneumoniae* Friedländer, and *B. morgan* I all gave gas on occasions. Gas-formation occurred in 30 per cent. of the tubes inoculated with *B. paratyphosus* C and this represents the most constant result obtained with any organism on this salt.

As will be shown later, apparent gas-formation bears no constant relation to the decomposition of this salt, and changes in reaction during complete decomposition may be very slight.

*d-Tartrates.* With certain organisms the results obtained with these salts were very constant. *B. typhosus* gave an early and very marked acidity followed after 48 hours by alkalinity. There was no gas-formation. *B. paratyphosus* A gave practically no change in reaction and no gas. *B. paratyphosus* B a slight alkalinity with no preceding acidity and no gas. *B. paratyphosus* C gave a well-marked early acidity changing later to alkalinity and abundant

and constant gas-formation. *B. enteriditis*, Gärtner, Mutton, Newport, *B. suis-pestifer* and most of the other types behaved irregularly.

As a result of more than 4000 observations on reaction and aërogenesis, we found that whereas such changes in reaction as occur are reasonably constant, apparent aërogenesis is an even more variable factor with these salts than with the "sugars." Therefore as the reaction changes alone are insufficient to afford a basis for differentiation we were compelled to abandon the test in this form. Before doing this, however, every effort was made to determine the causes of irregularity in aërogenesis.

The following factors which might possibly influence aërogenesis were investigated:

(a) Method of sterilisation of the medium.

- (1) By autoclaving at 115° C. for 15 minutes.
- (2) By steaming on two successive days for 20 minutes each.
- (3) By filtration through a Doulton germ-proof filter candle.

(b) Reaction of the medium.

- (1) The effect of adjusting the medium to various reactions from pH 6.6 to pH 8.0 was investigated.
- (2) The medium was used "buffered" and "unbuffered."

(c) The nature of the inoculum.

- (1) Sowing from cultures on solid and in liquid media.
- (2) Sowing from broth cultures of varying age from 3 hours to several weeks.
- (3) Variation in the quantity of the inoculating dose.

(d) Variations in the organic salt medium.

- (1) Use of different concentrations of the fermentable salts.
- (2) Employment of a richer basal medium, including the use of different brands of peptone.
- (3) Omission of the indicator.

This formed a very extensive piece of work, which yielded many observations of interest, but, unfortunately, for its main object, the investigation of variability in aërogenesis, it proved fruitless (cf. however p. 8).

In a previous paper on this subject one of the present authors (Brown, 1921) showed that the growth of certain bacteria was inhibited while that of certain others was enhanced by the addition of a soluble citrate to the medium, and that where enhancement of growth occurred, the citrate was decomposed and utilised by the organisms. This fact could be easily demonstrated by the addition of a solution of *lead acetate* to the cultures, when those tubes which showed enhancement of growth yielded a small granular precipitate, while those which showed inhibition yielded a very bulky white precipitate of lead citrate similar to that formed in the control tube.

Koser (1923, 1924), using a large number of strains of the colon-aërogenes

group, has confirmed these observations on the value of inhibition or enhancement of growth in citrated media as a differential test for these organisms. He does not, however, employ the method of precipitation by lead salts.

The question then arises as to whether the precipitation test can yield more consistent results than those obtained by recording acid or alkaline changes and aërogenesis. In this connection a strain of *B. suispestifer*, which gave a feeble fermentation of dulcitol was chosen and plated out. Twelve discrete colonies were picked off into a series of broth tubes, and from these twelve dulcitol tubes were sown. Of the twelve, four showed vigorous fermentation with a copious evolution of gas and the remaining eight were unaffected. These twelve cultures were then tested on different salts of *d*-tartaric acid, but here again gas formation was a variable phenomenon occurring in only seven out of twenty-four tubes, although all showed an acid change followed by alkalinity. When, however, the precipitation test was applied to these twenty-four tubes, decomposition was found to be complete in every case. Examination showed the cultures to be pure, and the agglutination reactions (with *B. paratyphosus* C and *B. glässer* sera) were normal.

Similar observations were made on these and other salts with strains which were notoriously irregular in their reaction changes and aërogenesis, and in all cases the precipitation test was found to yield consistent results.

As further evidence of the superiority of the precipitation method we would cite the fermentation of salts of *l*-tartaric acid. These salts may be completely decomposed by various organisms with no other external indication of the changes taking place than luxuriance of the bacterial growth.

We are satisfied that aërogenesis is not a satisfactory criterion and that determination of decomposition of the salts by the precipitation method is a rational and trustworthy test.

#### *Technique of the Precipitation Test.*

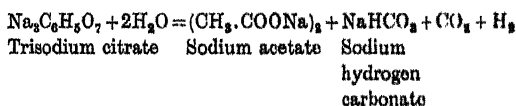
As it was found that commercial meat extracts yield appreciable precipitates with soluble lead salts, it became necessary to abandon nutrient broth as a basal medium, and to employ peptone water. Various commercial peptones were investigated and the most satisfactory results were found to follow the use of "Bactopeptone," which possesses the advantage of being approximately neutral in reaction.

The organic salts are employed in a concentration of 1 per cent. in peptone water (1 per cent. bactopeptone). The reaction of the medium is corrected, if necessary, to pH 7.4. The medium is then tubed in 5 c.c. quantities and autoclaved at 115° C. for 20 minutes. Inoculation is made from 24 hours old broth cultures, a 3 mm. platinum loop being employed and the cultures are incubated at 37° C. for 48 hours. For precipitation a saturated solution of lead acetate is employed in the following proportions: 0.4 c.c. is added to 5 c.c. of the citrate medium, and 0.6 c.c. to 5 c.c. of the tartrate or fumarate (p. 12) medium. The tubes are shaken to ensure thorough mixing and are then

replaced in the rack to sediment; they may be examined in a few hours or preferably next day. When decomposition of the salt has not occurred, a voluminous white flocculent precipitate of the lead salt is formed. This precipitate does not settle to the bottom but tends to remain in suspension indefinitely. On the other hand, when decomposition has occurred, a small heavy granular precipitate, made up chiefly of lead carbonate forms, and slowly settles to the bottom of the tube as a small greyish deposit: the colour varying depending on the nature and extent of the impurities (chiefly traces of sulphates and ferric oxide and ferrous sulphide) derived from fermentation of the basal medium.

As the decomposition of citric acid is the most interesting of the series of reactions, a more detailed examination has been made of the products formed from sodium citrate in a typical case, viz. fermentation of sodium citrate by *B. swipestifer*. Apart from traces of the coloured impurities referred to above, only three products were found, viz. acetic acid, carbon dioxide and a trace of succinic acid.

The amount of acetic acid was determined by making 100 c.c. (= an original content of 1 gramme of trisodium citrate) of the reaction liquid, acid, with sulphuric acid and steam-distilling the mixture until 10 c.c. of the distillate did not require more than 0.1 c.c. of *N*/5 barium hydroxide solution for neutralisation, and then titrating the total distillate. The mean of several estimations gave 0.48 gramme of acetic acid per 100 c.c. which corresponds with the formation of two molecules of acetic acid (0.46 gramme per gramme) from one molecule of trisodium citrate, thus



The results, which would be expected to be low, were always somewhat high and this was traced to the action of the organism on the bacto-peptone solution. A control solution free from citrate, but otherwise prepared like the citrate solution, gave from 100 c.c. an acid distillate corresponding to 0.09 gramme. When the acid distillate from the action of the organism on citrate in bacto-peptone water is corrected by this amount it will be seen that the average result 0.39 gramme from 100 c.c. is not far from the amount, 0.46 gramme, required by the equation.

The distillate, after neutralisation by baryta, was taken to dryness and the residual salt crystallised in three fractions, which on analysis gave the following results: Barium, per cent. I, 53.88; II, 53.61. The third fraction was converted into the silver salt and this, after recrystallisation from hot water, gave on ignition silver 64.9 per cent. Barium acetate,  $\text{Ba}(\text{C}_2\text{H}_3\text{O}_2)_2$ , requires Ba 53.81 per cent. and silver acetate,  $\text{AgC}_2\text{H}_3\text{O}_2$ , requires Ag 64.66. The whole of the salts obtained from the volatile acids were used in this series of determinations so that there is no room for doubt that the sole volatile acid formed is *acetic acid*.

One hundred c.c. of a 1 per cent. solution of trisodium citrate on precipitation by saturated lead acetate yields 1.51 grammes of lead citrate,  $\text{Pb}_3(\text{C}_6\text{H}_5\text{O}_7)_2$ , dried at  $110^\circ\text{C}$ . *in vacuo*. After the action of *B. suispestifer* on a similar solution of sodium citrate in bactopectone water, the lead precipitate from 100 c.c. of solution weighed only 0.6452 gramme, and this contained 7.62 per cent. of carbon dioxide as estimated directly in a Schrotter apparatus, corresponding to 0.049 per cent. of carbon dioxide present in the fermented liquor as sodium bicarbonate. The carbon dioxide in the solution was then estimated independently by precipitation with saturated calcium chloride.

One hundred c.c. of the liquor gave no immediate precipitate with the reagent, but did so on boiling, indicating as was to be expected, that the carbon dioxide was present as sodium bicarbonate. The amount of calcium carbonate formed was 0.1204 gramme.

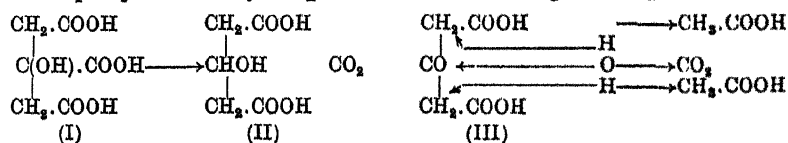
A second 100 c.c. was then made alkaline with ammonia solution and precipitated by calcium chloride solution, yielding 0.2674 gramme of calcium carbonate. These two precipitates were mixed, and carbon dioxide in the mixture estimated: it amounted to 38.7 per cent. Calcium carbonate requires 44.0 per cent. So that the calcium carbonate, like the lead carbonate, contains impurities. This corresponds to 0.103 gramme of carbon dioxide in 100 c.c. of fermented liquor as bicarbonate. The equation given above requires 0.16 gramme. The deficiency is, no doubt, due to the liberation of carbon dioxide from the bicarbonate primarily formed from the citrate by the acids formed by the fermentation of the bactopectone.

A quantity (750 c.c.) of the fermented liquor was distilled to yield 300 c.c. of distillate. The latter was then re-distilled, after making distinctly acid with sulphuric acid, to yield 100 c.c. of distillate. This had specific gravity 0.9993 at  $15^\circ/15^\circ$ ; on re-distillation to yield 50 c.c. the specific gravity fell to 0.996 at  $15^\circ/15^\circ$  and the distillate contained traces of insoluble fatty acids. It was then re-distilled, after the addition of potash solution, to yield 30 c.c., which had specific gravity 0.996 at  $15^\circ/15^\circ$  and gave a slight iodoform reaction. A blank test with bactopectone water fermented by the same organism, carried out in precisely the same manner yielded a final distillate having specific gravity 0.994 at  $15^\circ/15^\circ$ , so that whatever the trace of volatile neutral substance produced, causing the fall in specific gravity, may be, it appears to come from the bactopectone and not from the citrate.

The whole of the mother liquors from the foregoing estimations were kept, de-leaded when necessary, and neutralised. They were then taken to low bulk *in vacuo* until salts, chiefly sodium sulphate from the reagents used, began to crystallise out. The mother liquor from these was made acid with sulphuric acid and thoroughly extracted by agitation repeatedly with ether and acetic ether. The solutions in these solvents were dried over anhydrous sodium sulphate and the solvents distilled off leaving sticky residues, which gradually became crystalline. The residues were mixed, boiled with water till nothing more dissolved, the boiling solution decolourised with charcoal,

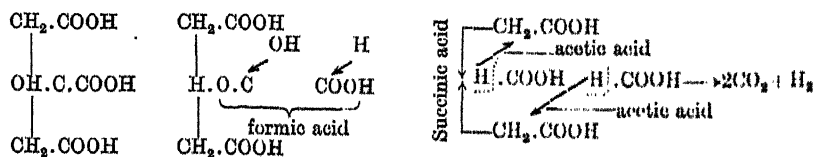
filtered and concentrated to low bulk, and left to crystallise. The crystalline acid obtained melted at  $180^{\circ}$ , had a molecular weight of 120 (if dibasic) as determined by titration with  $N/5$  baryta solution, and yielded a beautifully crystalline anhydrous barium salt, which on analysis furnished 92.75 per cent. of barium sulphate. Succinic acid melts at  $181^{\circ}$ , has a molecular weight of 118, and barium succinate is anhydrous and should yield 92.11 per cent. of barium sulphate. The amount of pure succinic acid obtained from liquors originally containing 10 grammes of trisodium citrate was only 0.0786 gramme, but owing to the greasy and intractable character of the ether and acetic ether residues, it was probably not all recovered.

A control experiment with similar quantities of bactopectone water fermented by the same organism yielded under like treatment no crystalline acids, though it yielded to ether and acetic ether residues very similar in character to the crude residues obtained from the fermented citrate liquor. It seems clear from these results that sodium citrate (citric acid I) is fermented by *B. suispestifer* in a very simple manner, which might be represented thus:



If this were the case we should have expected to find traces of  $\beta$ -oxyglutaric acid (II) or acetonedicarboxylic acid (III) in the fermentation liquor, but the only acid found apart from the two ultimate decomposition products was succinic acid. In view of this we think it more probable that the group

$\text{C(OH).COOH}$  in citric acid is attacked at once and as a whole by the organism giving rise by hydrolysis to two molecules of formic acid and momentarily to two  $\text{CH}_2\text{COOH}$  residues. The formic acid is then decomposed by the organism liberating hydrogen and carbon dioxide, the latter partly escaping and partly forming sodium bicarbonate, whilst the hydrogen combines with the  $\text{CH}_2\text{COOH}$  residues to form acetic acid, but the latter reaction is not quite complete, and part of the  $\text{CH}_2\text{COOH}$  residues coalesce to form succinic acid<sup>1</sup>, thus:



If this explanation is correct it throws some light on the variability of aërogenesis, since the amount of hydrogen evolved will depend on the amount of

<sup>1</sup> While this paper was being prepared, a paper by Grey (1924) appeared in which a similar explanation is given of the decomposition of citric acid by *B. coli*.

succinic acid formed, and where conditions favour the production of this acid, the amount of hydrogen evolved will be considerable, whereas if the production of acetic acid is favoured the amount of hydrogen liberated will be small.

### *Salmonella Group.*

Owing to the increasing importance of the *Salmonella* group of bacteria, evidenced by the mass of literature, which has accumulated during the past few years, and the present divergence of opinion on the classification of the various members of the group, we considered that it would form suitable material for the trial of this new test. For this purpose we employed the following *Salmonella* strains:

<i>B. paratyphosus</i> A	5 strains	<i>Salmonella</i> , Stanley type	2 strains
<i>B. paratyphosus</i> B	13 "	" Reading "	1 strain
<i>B. paratyphosus</i> C	11 "	" Binns "	2 strains
<i>B. suispestifer</i>	12 "	" "G" "	2 "
<i>B. aertrycke</i> , Mutton type	10 "	<i>B. völdägsen</i>	3 "
" Newport type	2 "	<i>B. glässer</i>	2 "
<i>B. enteritidis</i> , Gärtner	13 "		

We take this opportunity of expressing our thanks to Dr R. St John-Brooks, Curator of the National Collection of Type Cultures, for his great kindness in supplying us with the majority of these strains, and also to Sir Frederick Andrewes for several strains of *B. suispestifer*.

With the exception of some of the Gärtner strains, all the foregoing *Salmonellas* yielded perfectly constant results when tested on sodium citrate, sodium *d*-tartrate and sodium *l*-tartrate, and it will be shown that these results must prove of considerable help in allocating the various types to their respective positions within the group. The discrepancies shown by the Gärtner strains will be discussed later. The action of each type on these three salts may be summarised as follows:

*B. paratyphosus* A did not ferment any of the salts mentioned. The cultures therefore after the addition of the lead acetate solution gave a bulky white suspension similar to that in the control tube, indicating absence of decomposition.

*B. paratyphosus* B. All thirteen strains readily decomposed citrate and *l*-tartrate, but failed to decompose *d*-tartrate. Five strains of this type were grown on *d*-tartrate medium at 37° C. for 28 days, but when tested no evidence of decomposition of the salt could be found.

*B. paratyphosus* C. The eleven strains of this type differed markedly from *B. paratyphosus* B in their constant failure to ferment *l*-tartrate within 48 hours, while readily fermenting *d*-tartrate. They also fermented citrate.

*B. suispestifer*. The eleven strains gave reactions similar to those recorded for *B. paratyphosus* C. The same applies to the single strain of type Reading.

*Salmonella* Type "G." One strain of this type, and a strain isolated during the course of this work by blood culture from a case clinically resembling enteric fever and identified serologically with Type "G," were not distinguishable by fermentation of the three salts from *B. paratyphosus* C or *B. suispestifer*.



## The fermentation of Salts

*B. aertrycke*, Mutton. The eleven strains constantly decomposed all three salts. A similar result was obtained with the two strains of the Newport type, the two of type Binns, and two of type Stanley.

So far, in dealing with sixty strains of ten *Salmonella* types, we have met with perfectly consistent results, and before passing to the irregular behaviour of the *B. enteritidis*, Gärtner strains, it is convenient to summarise these results.

Organism and number of strains tested	Trisodium citrate	Sodium <i>d</i> -tartrate	Sodium <i>l</i> -tartrate
5 <i>B. paratyphosus</i> A	-	-	-
13 <i>B. paratyphosus</i> B	+	-	+
11 <i>B. paratyphosus</i> C	+	+	-
12 <i>B. suispestifer</i>	+	+	-
10 <i>B. aertrycke</i> , Mutton	+	+	+
2 <i>B. aertrycke</i> , Newport	+	+	+
2 <i>B. aertrycke</i> , Binns	+	+	+
2 Type Stanley	+	+	+
1 Type Reading	+	+	-
2 Type "G"	+	+	-
2 <i>B. glässer</i>	-	-	-
1 <i>B. völdägsen</i> (Damman)	-	-	-
1 <i>B. völdägsen</i> (Wegener)	+	+	+

(+ = decomposition, - = no change)

It has already been stated that these results were obtained after 48 hours' incubation and it may be asked whether any delayed reactions were observed. During the investigation of these sixty strains delayed reactions were observed twice, viz. *B. suispestifer* (Indiana) which failed to decompose citrate in 48 hours but decomposed it in 96 hours, and one strain of *B. aertrycke*, Mutton (MacConkey) which also took 96 hours to decompose citrate.

Plate I shows the results of an actual test with four different *Salmonella* types grown on three of the salt media (trisodium citrate, sodium *l*-tartrate, and sodium *d*-tartrate). It will be seen that those tubes in which decomposition of the salt has occurred show only a small greyish precipitate after addition of lead acetate, while those in which the salt was unaffected show a bulky white suspension.

### Irregular behaviour of some Gärtner strains.

We have examined the following thirteen strains: the numbers refer to the catalogue of the National Collection of Type Cultures:

<i>B. enteritidis</i> Gärtner (Bainbridge)	75	<i>B. enteritidis</i> Gärtner (Guinea pig 158)	252
" (Limerick)	125	" (Turner)	305
" (Newcastle)	126	" (Rockefeller Inst.)	410
" (Stokes)	127	" (Paracoli)	577
" (McNee)	128	" (Ratin)	617
" (Original)	203	" (Liverpool virus)	618
" (Danzysz)	205		

Of these strains Nos. 127, 203, 252, 305, and 410 decomposed all three salts in 48 hours behaving thus like the *aertrycke* strains. The remaining strains exhibited considerable irregularity in their action on dextro- and levo-tartrates; sometimes fermenting these salts completely in 48 hours, but more

frequently requiring 96 hours or longer, and occasionally failing altogether. Two strains (126 and 128) rarely attacked these two salts.

When decomposition did not occur, it was noticed that the reaction of the medium became slightly acid and remained so. In complete decomposition an early acid change was always succeeded by a marked alkaline change probably due to the production of sodium bicarbonate from the fermentation of the salt.

Even in simple peptone water these types produce an alkaline reaction in about 48 hours.

Examination showed the cultures of all strains to be pure. Agglutination and absorption tests were normal and specific except with strains 127 and 410, which were "rough." Sugar fermentations were perfectly normal.

The initial reaction of the medium influenced decomposition to some extent. Thus, at an initial reaction of  $pH$  8.0 six of the strains failed to decompose dextro-tartrate in 96 hours, although this reaction is not unfavourable to fermentations by other types; at  $pH$  7.7 three strains failed, and at  $pH$  7.4 only two strains failed. No further adjustment of reaction gave any more favourable result. Attempts were made to enhance the fermenting powers of the "defective" strains by various means, but were not successful. A richer basal medium was employed and various factors, already discussed in connection with irregularity in aërogenesis, which we thought might influence decomposition of tartrates, were examined, but no explanation of the irregular results was forthcoming. One observation is, however, worth recording. The strains which utilised the two salts irregularly or not at all appeared to be of more delicate habit than those which fermented vigorously and constantly: they died out more quickly in culture. When a set of three months old agar cultures of the thirteen strains was examined, it was found that the only ones living were those which constantly fermented *d*- and *l*-tartrates: the irregular fermenters were dead. This inherent defect of the Gärtner strains was not observed in any other type.

In order to extend the range of usefulness of the precipitation test by adding to the number of test salts, all the salts of organic acids previously employed in the tests for aërogenesis and reaction changes were examined to determine which were suitable for the precipitation technique. It was found that the following formed insoluble lead salts similar to those of citric and tartaric acids: malic, malonic, mesotartaric, racemic, pimelic, aconitic, and maleic acids.

Fumaric, succinic, glutaric and glycollic acids give small heavy granular precipitates with soluble lead salts.

Of these salts, malonate, pimelate, succinate, aconitate, glutarate, and glycollate were apparently not readily decomposed by any member of the *Salmonella* group or allied types.

Racemate, which is composed of an equal moiety of dextro-tartrate and of laevo-tartrate, was decomposed to the extent of 50 per cent. by those types which affected either the laevo- or dextro-salt only. It was completely de-

composed by those types which ferment both varieties, and was unaffected by those which ferment neither. Its use was not continued as more information could be obtained by using the dextro- and laevo-salts separately.

*Fumarate.* The reactions with this salt were of special interest and of some value. Lead fumarate forms a relatively small characteristic precipitate crystallising in slender needles which sinks rapidly to the bottom of the tubes. When, however, a fumarate medium (1 per cent. sodium fumarate in peptone water) was sown with various types of the *Salmonella* group, it was found on precipitating the cultures after 48 hours' growth that all the tubes showed a voluminous white precipitate, which remained in suspension, occupying more than three-quarters of the liquid column. The appearance of the tubes was therefore the reverse of that seen in the fermentation of the salts hitherto examined; the cultures in which change had occurred giving a bulky white suspension while the control tube showed a small granular precipitate. When the cultures on fumarate were incubated for four days a second phase of fermentation occurred and when precipitation was done at the end of this phase, it was found that the bulky suspension appeared only in the cultures of *B. paratyphosus* A and *B. paratyphosus* C; all the other types giving eventually the slight greyish precipitate consisting chiefly of lead carbonate indicating still further change. The value of this test lies in distinguishing the "C" type from *B. suispestifer*, Type Reading and Type "G."

In view of the interesting character of the action of these bacteria on sodium fumarate, an investigation has been started into the nature of the decomposition products formed. The fermented liquors were examined in the following way, and the results are given in the Table on p. 13.

One hundred c.c. of the fermented liquor were precipitated by excess of saturated lead acetate solution, and the precipitate collected, washed, dried and weighed (line 1). In it the amount of lead (line 2) was determined: it included lead carbonate in all cases. One hundred c.c. of the fermented liquor were made acid with acetic acid, boiled to remove carbon dioxide, neutralised with ammonia and then precipitated with lead acetate and the precipitate collected, washed, dried and weighed (line 3) and the lead in it determined (line 4).

The amount of carbon dioxide present was estimated by adding saturated calcium chloride solution to 100 c.c. of the fermented liquor, previously rendered alkaline with ammonia, and the precipitated calcium carbonate collected, washed, dried and weighed (line 5). As it frequently contained other calcium salts, the carbon dioxide in it was determined (line 6), and from this the percentage of carbon dioxide present in the solution calculated (line 7). These figures are not satisfactory, as the amount of "calcium carbonate" precipitated varied with the time of standing, *e.g.* from 0.2160 gramme in 12 hours to 0.775 gramme in 14 days from *B. aertrycke*, Mutton, 96 hours, liquor. The figures given are for 12 hours. The volatile acids (line 8) were determined by distilling 100 c.c. of the liquor after making acid with sulphuric acid.

Finally, from the mother liquors available from these determinations, the organic acid or acids present (line 9) were isolated by de-leading when necessary, neutralising with soda, concentrating to very low bulk and, after acidifying with sulphuric acid, extracting with ether.

The crude acids so isolated were crystalline, except in the case of *B. paratyphosus* C 90; here they were partly crystalline and only the crystalline portion was used for the examination. These crude acids were neutralised with soda (line 9, I (a)), the solution taken to dryness and boiled with absolute alcohol to obtain a clean sodium salt, which was then converted into the lead salt by precipitation with lead acetate (line 9, I (b)), and from the lead salts the acids were recovered and re-examined (lines 9, II (a), (b), (c)).

	<i>Bacillus aertrycke</i> , Mutton			<i>Bacillus</i> <i>swipestifer</i> Krause 412	<i>Bacillus</i> <i>paraty-</i> <i>phosus</i> C 90	<i>Bacillus</i> <i>paraty-</i> <i>phosus</i> A
	24 hrs	48 hrs	96 hrs	96 hrs	96 hrs	96 hrs
1. Lead ppt. from 100 c.c. fermented liquor, grammes	1.73	1.37	1.6	0.87	1.6	1.51
2. Lead, % in 1 ... ..	68.4	68.4	68.1	71.6	66.1	64.6
3. Lead ppt. from 100 c.c. of fermented liquor freed from carbonates, grammes ... ..	1.17	0.92	0.32	0.59	0.28	1.15
4. Lead, % in 3 ... ..	63.4	67.9	68.3	72.3	61.6	71.2
5. Calcium carbonate ppt. from 100 c.c.* ... ..	0.084	0.178	0.216	0.301	0.231	0.112
6. Carbon dioxide, % in 5* ... ..	—	33.0	31.6	43.9	37.0	30.9
7. Carbon dioxide in 100 c.c. of fermented liquor, grammes (Calc. from 6)* ... ..	—	0.06	0.07	0.13	0.08	0.03
8. Volatile acids from 100 c.c. expressed as acetic acid, grammes ... ..	0.03	0.03	0.03	0.11	0.04	0.04
9. Characters of crude crystalline acids recovered from fermented liquor†						
I. (a) Soda to neutralise, % ...	68.5	60.0	56.3	58.6	64.7	56.8
(b) Lead in lead salt, % ...	63.4	64.6	64.5	64.5	66.4	65.7
Acids as recovered from lead salts						
11. (a) Melting point ... ..	291°	Two fractions m.p. 291° and m.p. 285°	181°	181°	181°	285°
(b) Soda to neutralise, %	68.5	66.0	65.7	66.2	—	64.9
(c) Lead in lead salt, % ...	64.8	64.4	—	64.5	65.1	64.3
10. Composition of recovered crystalline acid ... ..	fumaric	fumaric	succinic	succinic	succinic	fumaric

\* These figures are unsatisfactory as the amount of "calcium carbonate" precipitated varied with the time of standing: the time selected was 12 hours.

† Fumaric acid melts at 291°, requires 68.9 % of soda for neutralisation, and the lead salt contains 64.5 % of lead.

Tartaric acid melts at 170°; racemic acid at 205–6°. Both require 53.3 % of soda for neutralisation, and the lead salts contain 53.3 % of lead.

Succinic acid melts at 181°, requires 67.7 % of soda for neutralisation, and the lead salt contains 64.09 % of lead.

For determining the nature of the acid isolated (line 10 in Table) the quantities of pure acid ultimately obtained were so small that the method of mixed melting points only could be used. It appears from these results that of the four organisms dealt with, all except *B. paratyphosus* A decompose



acetate yields a small precipitate of lead carbonate. These two precipitates can be easily differentiated by adding a little acetic acid, which dissolves the lead carbonate but not the lead mucate. The technique of the precipitation test must therefore be modified in the case of this salt by the addition of glacial acetic acid to the lead acetate solution: the proportions employed by us are—glacial acetic acid 0.4 c.c. and saturated solution of lead acetate 0.6 c.c. to 5.0 c.c. of the mucate culture. If the mucate is unaltered, a dense turbidity settling down to a small precipitate results, but if decomposition of the salt has occurred, no precipitate follows the addition of the acid lead acetate solution.

The following types constantly decomposed the 1 per cent. sodium mucate medium, *B. paratyphosus* B, Mutton, Newport, Stanley, Binns, Reading and Gärtner. It was not decomposed by *B. paratyphosus* A, *B. paratyphosus* C, *B. suispestifer*, or Type "G." It therefore offers a means of distinguishing the Reading type from *B. suispestifer* and Type "G." We observed no irregularities in the fermentation of this salt by the various types.

It will be seen from the foregoing that with the use of six salts: citrate, *d*-tartrate, *l*-tartrate, *m*-tartrate, fumarate, and mucate, we have succeeded in obtaining seven different cultural groupings from the eleven different *Salmonella* types. The types we have still failed to differentiate are Mutton, Newport and Binns from one another, and *suispestifer* from Type "G."

The following table gives a summary of the reactions with these six salts, and it is convenient at this stage to compare these results with those hitherto obtained from "sugar" fermentations.

	Citrate	<i>d</i> -tartrate	<i>l</i> -tartrate	<i>m</i> -tartrate	fumarate	mucate
<i>B. paratyphosus</i> A	—	—	—	.	.	—
<i>B. paratyphosus</i> B	+	—	+	.	.	+
<i>B. paratyphosus</i> C	+	+	—	.	—	—
<i>B. suispestifer</i>	+	+	—	.	+	—
<i>Salmonella</i> type "G"	+	+	—	.	+	—
Type Reading	+	+	—	.	+	+
Type Mutton	+	+	+	+	.	+
Type Newport	+	+	+	+	.	+
Type Binns	+	+	+	+	.	+
Type Stanley	+	+	+	—	.	+
<i>B. enteritidis</i> , Gärtner	+	±	±	±	+	+

Results were taken at the end of 48 hours' incubation at 37° C. except in the case of fumarate, in which the incubation was prolonged to 96 hours to allow for its slower decomposition. A + sign indicates complete decomposition of the salt as evidenced by the formation of only a small precipitate consisting chiefly of lead carbonate on the addition of the lead acetate solution. A — sign indicates failure to decompose as evidenced by a bulky precipitate of the lead salt of the undecomposed acid. In the case of fumarate a — sign indicates change of the salt as indicated by a bulky precipitate on addition of lead acetate (cf. p. 12) while a + sign expresses further change as shown by a small precipitate of lead carbonate. For the modified precipitation technique applied in the case of mucate, see above.

The grouping of the types from the above table is as follows: (1) *B. paratyphosus* A, (2) *B. paratyphosus* B, (3) *B. paratyphosus* C, (4) *B. suipestifer* and Type "G," (5) Reading, (6) Mutton, Newport, Binns and Gärtner?, (7) Stanley.

*Differential "Sugar" Fermentations of the Salmonellas.*

	Xylose	Arabinose	Dulcitol	Inositol
<i>B. paratyphosus</i> A	-	+	+	-
<i>B. paratyphosus</i> B	+	+	+	+
<i>B. paratyphosus</i> C	+	+	+	-
<i>B. aertrycke</i> , Newport	+	+	+	-
<i>B. aertrycke</i> , Mutton	+	+	+	+
<i>B. enteritidis</i> , Gärtner	+	+	+	-
<i>B. columbensis</i>	+	+	+	-
<i>B. suipestifer</i>	+	-	-	-

(Andrews and Neave, *B.J.E.P.*, 1921.)

These "sugar" reactions give us four groups: (1) *B. paratyphosus* A, (2) *B. paratyphosus* B and Mutton, (3) *B. paratyphosus* C, Newport, Gärtner, and *B. columbensis*, (4) *B. suipestifer*.

It possesses an advantage over the organic salts table in differentiating Mutton from Newport, and if the two tables are combined we are presented with eight groups.

We are not aware of the reactions of the newer types Stanley, Binns, Reading, and "G" with the rarer "sugars" nor are we in a position to determine these, but we have no information that they would be helpful in differentiating these types from one another or from other types.

*The optimum time limit of incubation.*

Although we have shown that six strains of *B. paratyphosus* B taken at random failed to decompose *d*-tartrate after 28 days' incubation, this does not hold good in the case of the laevo-salt when acted upon by *B. paratyphosus* C, for, although no apparent decomposition occurs within the first 48 hours, several strains of this organism were found capable of fermenting this salt when incubation was prolonged. This is directly supported by the observations of Pasteur, who showed that when *Penicillium glaucum* was grown in solution of ammonium *dl*-tartrate the salt of the dextro-acid was destroyed and that of the laevo-acid remained; if, however, the decomposition was allowed to proceed further, the laevo-salt was also destroyed. It is obvious, therefore, that when using laevo-tartrate a definite time limit must be adhered to, and we have found 48 hours the most suitable.

Although prolonged incubation does not affect the value of the test in the cases of citrate and *d*-tartrate, for the sake of uniformity we advise a period of 48 hours, which, in practically all cases, is sufficient; this also applies in the cases of mesotartrate and mucate, but with fumarate, in which the action occurs more slowly and may take place in one or more stages (see p. 14), it is necessary to prolong the incubation to 96 hours.

*Combinations of fermentable substances.*

Various mixtures of salts or of salts and sugars were examined. One of the most useful of these was a mixture of sodium citrate 1 per cent. and glucose 1 per cent., which was fermented by all of the group except *B. paratyphosus* A and C, *B. suispestifer* and Type "G," giving results similar to those obtained with sodium mucate, for which salt it might be substituted in an emergency.

The action of two organisms, viz. *B. paratyphosus* C 90 and *B. aerogenes*, on this mixture was investigated and compared with their action on the two components, sodium citrate and glucose separately. For this purpose the volatile acid (acetic acid) and the reducing power (glucose) were determined in the fermentation liquors; the results are given in the following table. The figures are for 100 c.c. of the fermented liquor in each case.

Organism	Medium	(Glucose % from reducing power)	Volatile acid % distillation and titration	Glucose % decomposed
<i>B. aerogenes</i> 24 hours	1 % glucose,	0.32	0.15	0.68
	1 % bactopeptone			
	1 % sodium citrate,	0.15	0.62	0.85
	1 % glucose,			
	1 % bactopeptone			
	1 % sodium citrate,	Nil	0.76	—
<i>B. paratyphosus</i> C. 90 24 hours	1 % glucose,	0.70	0.07	0.30
	1 % bactopeptone			
	1 % sodium citrate,	0.31	0.13	0.69
	1 % glucose,			
	1 % bactopeptone			
	1 % sodium citrate,	Nil	0.26	—
	1 % bactopeptone			

It is clear that with both these organisms more glucose is decomposed in presence of sodium citrate, whilst the decomposition of the citrate (as measured by the volatile acid produced) is retarded in presence of glucose. For a full explanation of these differences it will probably be necessary to determine the nature and amounts of the products formed by the action of the two organisms on glucose and citrate separately, but it seems possible that the differences are due to (1) increase in the nutritive material available leading to enhanced growth of the organisms, (2) preference of the organisms for glucose as a nutritive material, (3) alteration in the pH of the medium due to the fermentation products of glucose, which would tend to diminish the formation of acid products, e.g. the acetic acid produced by the decomposition of citric acid.

The addition of glucose to the dextro-tartrate medium appeared to prevent the decomposition of this salt by any member of the *Salmonella* group. The reasons suggested for the retarded decomposition of citrate by *B. aerogenes* and *B. paratyphosus* C 90 in presence of glucose probably also apply in this case, the similarity in decomposition products possibly having special importance in this instance.

Various mixtures of two or of three salts, giving a total concentration of 1 per cent. were tried, and in some cases yielded interesting results which,



however, need not be detailed here, as they do not bear on the main question of differentiation.

In many instances combinations of two salts which individually were readily fermented by an organism gave a mixture which resisted decomposition by this organism. In other cases a salt not normally fermented by a certain strain was rendered susceptible to decomposition by the addition of a readily fermented salt.

The results obtained were so varied that at one time it was hoped by the use of such mixtures distinctive tests could be found for every type of the *Salmonella* group, and other groups, but although it was found that the reactions with individual strains of a type were remarkably constant, when several strains of a type were tried divergent results were obtained, and this form of test had to be abandoned. As an example of the use of "mixtures." Sodium formate and sodium citrate are both readily decomposed by *B. paratyphosus* C. If, however, these salts be combined in the proportion of 0.5 per cent. each, a strain of *B. paratyphosus* C will apparently still decompose the formate fraction but not the citrate. If, however, 0.5 per cent. sodium racemate be added to the formate-citrate mixture, complete decomposition of all three salts by this strain of *B. paratyphosus* C occurs.

We think these apparent anomalies are also explicable on the lines suggested above, but until we have examined the fermentation products in each case we prefer not to attempt a detailed explanation.

*The correlation of luxuriance of growth of the organism  
with decomposition of the salt.*

In the case of citrate, it has been shown (Brown, 1921) that decomposition and utilisation of the salt are associated with enhancement of growth of the organism, and that failure to utilise the salt is associated with a definite inhibition of growth. This enhancement or inhibition of the growth of different types by citrate has also been employed by Koser (1923, 1924) in his tests for the identification of the bacterial types found in domestic water supplies. The test, although not so distinctive as lead precipitation, is trustworthy enough when restricted to citrate, *d*-tartrate and one or two other salts which undoubtedly inhibit the growth of types incapable of decomposing them, but it cannot be depended upon with all salts as in many cases failure to decompose the salt is not associated with any apparent inhibition, and the utilisation of the salt does not in all cases lead to obvious enhancement of growth.

*Practical application of the precipitation method.*

In order to test the reliability of this means of differentiating the various members of the *Salmonella* group, Dr Schütze very kindly gave us eleven cultures which had presented certain difficulties as regards their serological classification, and the results which we obtained after 96 hours' incubation are shown in the following table.

Organism	Trisodium citrate	Sodium dextro-tartrate	Sodium laevo-tartrate
Kruse	+	+	+
Shanks	+	-	+
Lister	+	+	+
Mudd	+	+	-
Leeds	+	+	-
Edinburgh	+	+	+
<i>Aertrycke</i> Bainbridge	+	+	+
<i>Aertrycke</i> Kral	+	+	+
<i>Aertrycke</i> Lab.	+	+	+
Piper I	+	-	+
Piper II	+	-	+

A 96 hours' incubation was used in this case, as it was found that two cultures, viz. Kruse and Edinburgh, failed to decompose citrate in 48 hours. From these results it will be seen that Shanks, Piper I and Piper II gave the reactions of *B. paratyphosus* B; Mudd and Leeds behave similarly to *B. paratyphosus* C, *B. suispestifer* or Type "G," and that the remainder correspond to *B. aertrycke*. These results entirely agree with Dr Schütze's serological diagnosis as far as it was able to be established.

Two of the above strains are of special interest, viz.: (1) The organism Kruse was found by Dr Schütze (1920) to belong, by virtue of its agglutino-genic and absorptive properties, to a very definite *aertrycke* group, but differed from all its fellow members in not reducing fuchsin and not producing sulphuretted hydrogen in lead acetate medium; also it did not produce gas in dulcitate, however; on the organic salts it behaved as an *aertrycke*. (2) Piper I. When studying this organism, Dr Schütze (1920) found that it was only after preparing a Piper I serum and carrying out an inverse or mirror absorption test that the fact was revealed that it was a true *B. paratyphosus* B, and as we have seen with the organic salts, it behaves as such. So far we have confined our attention to the results obtained with the Salmonella group and we will now briefly discuss the value of this test in differentiating other bacterial groups.

#### *Vibrio cholerae and allied vibrios.*

Brown (1921) showed that the character of the growth in citrated broth was an easy way of distinguishing the vibrios of Metchnikoff and Finkler-Prior from the cholera vibrios pathogenic to man.

By applying the precipitation test we have found that the following vibrios readily decompose trisodium citrate, viz. *V. cholerae*, *V. El tor*, *Paracholera* A and B (Mackie and Storer), *Vibrio K.* and *Vibrio Forrest*, whereas the vibrios of Metchnikoff and Finkler-Prior have apparently no action on this salt.

#### *The colon-aërogenes group.*

Reference has already been made to the extensive work of Koser (1923-24) on the action of a very large number of strains of this group on citrated media.

We have found that with the strains which we have used both *B. lactis aërogenes* and *B. cloacae* are capable of readily decomposing citrate, thus

differing from *B. coli communis*, *B. coli communior* and *B. acidi lactici*, which fail to attack this salt.

The only other organic salt which we have found useful with this group is sodium mucate, which is apparently not decomposed by *B. cloacae*, but is by all the other members of this group.

#### *The diphtheria group.*

The only salt which enabled us to distinguish Hofmann's bacillus from the Klebs Loeffler's bacillus was sodium fumarate; with this salt the former organism, on the addition of lead acetate, yielded a precipitate similar to that in the control tube, whereas in the case of both virulent and avirulent diphtheria cultures a considerably larger precipitate was produced.

#### *B. mallei and B. whitmori.*

It was more especially when using sodium fumarate that differences were observed between the growth of these two organisms; two strains of the former and four of the latter were used.

*B. mallei* on this medium showed a very feeble growth, but in the case of *B. whitmori* a very turbid growth appeared in 24 hours and pellicle formation was marked in 48 hours. It should be mentioned that the four strains of *B. whitmori* had become rugose in character.

The precipitation test demonstrated a certain amount of decomposition of this salt by *B. whitmori*, but no such change took place with *B. mallei*.

Plate II shows the difference in growth of these two organisms on this medium.

#### *The action of B. typhosus on organic salts.*

Somewhat conflicting results have been obtained when studying the behaviour of eight strains of *B. typhosus* on these organic salt media.

Three of the eight strains failed to decompose either citrate or dextro-tartrate, two of them fermented both these salts, and two failed to decompose citrate but decomposed dextro-tartrate.

It is interesting to note in this connection the observations of Mandelbaum (1912), who obtained a bacillus from the blood or faeces of more than fifty patients with clinical typhoid fever in Munich, which he named *B. metatyphi*. This bacillus resembled *B. typhosus* in all respects except that it produced alkali instead of acid in media containing glycerol.

With the strains of *B. typhosus*, which we investigated, the three which failed to ferment either citrate or dextro-tartrate produced only a trace of acidity in glycerol after 96 hours' incubation, whereas those which fermented both these salts rendered the glycerol medium distinctly acid in 24 hours. Further it is interesting to note that the three strains which failed to decompose these salts and had a very sluggish action on media containing glycerol were all obtained from the same epidemic and were all mild cases of enteric fever. Each of the eight strains was plated out and twelve colonies picked off into

broth. The resulting cultures behaved on the organic salt media in every respect as the parent strains. Agglutination and reciprocal absorption tests showed no differences amongst any of the strains except in intrinsic agglutinability.

*Examination of other allied types.*

We have failed to distinguish between *B. melitensis* and *B. abortus* (Bang.) and between *B. pestis* and *B. pseudotuberculosis rodentium*.

SUMMARY AND CONCLUSIONS.

(1) Reaction changes and production of gas in organic salt media are not sufficiently constant to form a reliable diagnostic criterion for the differentiation of bacterial types.

(2) The enhancement or inhibition of bacterial growth in such media in the majority of cases bears a direct relationship to the utilisation of the salt by the organism. This furnishes a useful differential test for certain organisms when citrates are used, but cannot be applied in the cases of all salts.

(3) The bacterial decomposition of the salts of those organic acids which form insoluble lead salts can be clearly demonstrated by the addition of suitable quantities of a solution of lead acetate to the culture.

(4) By the use of six organic salts, seven different groupings of the common *Salmonella* types can be obtained, whereas the sugar reactions have, up to the present, yielded only four different groupings.

(5) Regarding other groups of bacteria, the organic salts form an easy means of distinguishing between pathogenic and certain non-pathogenic vibrios, and between certain of the members of the *coli-aërogenes* group, and also between *B. diphtheriae* and Hofmann's bacillus, as well as between *B. mallei* and *B. whitmori*.

(6) The six organic salts employed in this test are relatively inexpensive, will stand sterilising by autoclave, and can be obtained with certainty in a state of purity much more readily than the rarer "sugars."

(7) The nature of the decomposition products of citric acid has been fully examined in the case of *Bacillus suispestifer*; it has been shown that the products are acetic acid, carbon dioxide and succinic acid, and a simple explanation of the mechanism of this reaction is put forward. In the case of fumaric acid a preliminary examination shows that the acid is converted into succinic acid probably by direct reduction. Maleic acid appears to behave in an analogous manner to fumaric acid. Further work on these acids is in progress.

(8) A large number of organic acid salts have been tried, but only the six suggested have given useful results. It appears that simple aliphatic monobasic and dibasic acids, with the exception of formic acid, are not decomposed readily by the bacteria investigated, and this is also true of monohydroxycarboxylic acids. Readiness of decomposition is first shown by the dihydroxydicarboxylic acids (tartaric acids), and appears to be at its best in the hydroxytricarboxylic acid (citric acid).

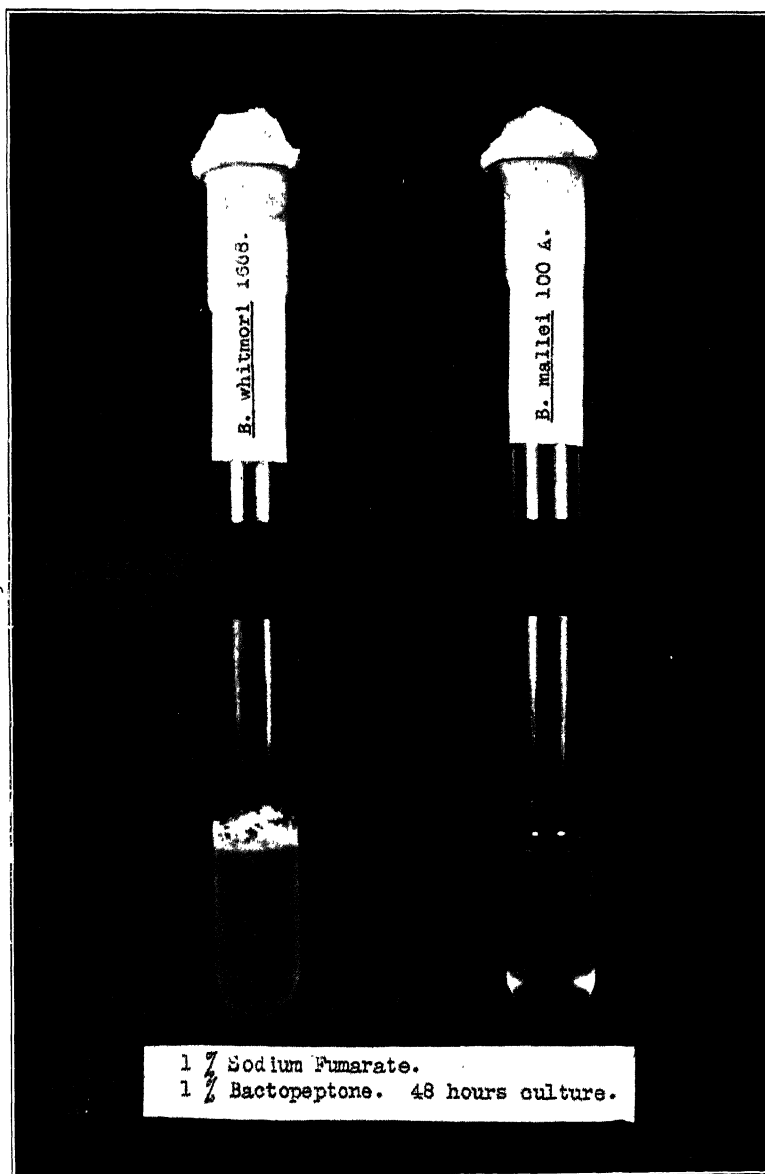
(9) While organic salt fermentation tests have been found particularly useful in the cases of the bacterial groups dealt with in this paper, they cannot be substituted for the "sugar reactions" in general use.

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# OBSERVATIONS ON THE GROWTH OF MENINGOCOCCI IN VITRO IN RELATION TO VIRULENCE.

A REPORT TO THE MEDICAL RESEARCH COUNCIL ON WORK CARRIED  
OUT AT THE UNIVERSITY OF CAMBRIDGE PATHOLOGICAL  
LABORATORY AND FIELD LABORATORIES.

BY E. G. D. MURRAY AND R. AYRTON.

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## I. INTRODUCTION.

EVERY bacteriologist is only too well aware of the many problems presented by the preparation of culture media for the growth of bacteria *in vitro*.

At present it is quite impossible to put forward a constructive generalisation outlining the principles of bacterial nutrition, based upon observed facts of bacterial metabolism.

A very large number of media have been described and certain of them are sufficient for the growth *in vitro* of many diverse bacteria; but the outstanding feature of our present degree of knowledge, in so far as pathogenic microbes are concerned, is the statement that each "species" behaves, to a greater or less extent, in a manner peculiar to itself.

It has long been recognised that special precautions have to be observed in order to obtain growth of certain parasitic bacteria and that the organism in question can be accustomed to grow upon what are described as the ordinary culture media in the course of a few subcultures. The question immediately arises, apart from the mere satisfaction of having obtained a culture of a given organism, whether these acclimatised bacteria may be regarded as possessing the physiological characters necessary to their parasitic existence. Our

investigation of this problem, as applied to the meningococcus, constitutes the main subject of this paper.

Murray (1924) emphasised the important relation of medium to the determination of the Minimal Lethal Dose of a meningococcus culture, but he purposely refrained from a detailed description of the medium he used in order that it might be considered in greater detail here.

There is another question which is of considerable importance to immunologists and which receives some attention in this paper, namely, the maintenance of meningococcal cultures *in vitro* over a sufficiently long period without loss of "virulence."

## II. THE ESTIMATION OF THE GROWING POWER OF A MEDIUM.

The literature abounds with so many different formulae, whereby it is recommended to make media for growing the meningococcus, that it is legitimate to suspect that a satisfactory medium yet remains to be discovered. Each entrant into this field of research finds cause for dissatisfaction in the existing formulae and proceeds to elaborate another. Still, that there are so many formulae indicates that the meningococcus can be grown quite readily and that a method is required for measuring and comparing the capacity of the various media to promote growth.

The method we have used for measuring the growing power of media during the last seven years is the following:

The surface of an agar medium, in Petri dishes, is inoculated with a chosen organism in a manner to ensure confluent growth. Convenient areas, as large as possible, are then ruled out on the glass surface of the plates and the growth over as many areas as possible is scraped off with a small sterile metal spade, made for the purpose; the growth so obtained is immediately placed in sterile weighing bottles with carefully ground stoppers and weighed. Then the stoppers are tilted and the bottles placed in a desiccator over NaOH, at reduced pressure and 37° C. and they are kept under these conditions until the weight becomes constant.

In this way the amount of moist living growth per sq. cm. surface of medium, the amount of dried bacterial protoplasm per sq. cm. and the percentage of moisture in the original growth are then easily computed. The usual precautions in weighing must be observed and the stoppers must be set in the bottles immediately the desiccator is opened because the dried bacteria are markedly hygroscopic.

We have frequently attempted to estimate, by naked eye comparison, the relative amounts of growth obtained on a number of different media, all inoculated at the same time and we have been somewhat astonished to observe how far removed from the truth our guesses were when they were compared with the actual weights of growth obtained from known areas. This in spite of a not inconsiderable experience of massive cultures of various organisms in the Army Vaccine Department during the war and elsewhere. We emphasise

this point in order to demonstrate that any naked eye method, whereby the amount of growth yielded by different media is to be estimated by looking at it *in situ*, is wholly untrustworthy. It has been our experience, that a very clear medium frequently gives a much more transparent growth of meningococcus than does some other medium not so completely freed of suspended matter, and in many such cases we have guessed that the more transparent growth was the lower yield per unit area, whereas actually the reverse was true. Thus we have come to the conclusion that the only reliable estimation of the amount of growth obtained on a medium, is one based upon actual measurement of the yield per unit area and the measure we have chosen is weight.

However carefully the various manipulations necessary to this measurement of growth are carried out, there is a considerable margin of error which is unavoidable at present. It is chiefly due to what may be called the adventitious moisture and this is determined very largely by the fluid exuded from the agar when it sets and the amount of water condensed when the plates cool. Both factors are difficult to control.

The amount of exudation moisture we regard as depending upon the firmness or rigidity of the jelly formed by the agar. It is our experience that a low concentration of agar means a moister surface when set than does a high concentration and this exudation moisture is in reality the nutritive medium and it contains a goodly proportion of solids. Other things being equal, there is a moderate degree of surface moisture most favourable to meningococcal growth and degrees of moisture varying markedly on either side of this diminish the amount of growth yielded. It may be that a too rigid jelly holds the nutrient solution too tenaciously for its ready accessibility to the micro-organism and that a too sloppy surface is approaching towards culture in a fluid medium.

The condensation moisture can be reduced by not using plates which expose a very large surface for condensation and by having the plates at a slightly warmer temperature than the medium at the time of pouring. Even so the lid and walls of the plate cool more quickly than the body of the agar and the condensation moisture is found to be very variable.

It might be suggested that the surface of the agar be dried in the usual way in the incubator but it must be remembered that agar readily forms a hard surface "skin" which is very unfavourable to meningococcal growth.

The error introduced by the inexact measurement of the area scraped can be diminished by scraping sufficiently large areas. With normal cultures the growth is removed very completely and the amount left behind is negligible compared to the possible error in the opposite direction which is introduced if care is not taken to avoid pressing on the surface and in so doing squeezing fluid out of the jelly.

In spite of these unavoidable errors the various measurements are sufficiently comparable to yield useful results and very considerable variations are revealed which, nevertheless, cannot be detected by the eye.

There is another point of importance which might easily be overlooked, if the amount of moisture the growth contains were not considered, that is the age of the culture at the time the growth is measured. If the yield of growth per unit area of the same medium is compared after 24 hours' and 48 hours' incubation, it is found that the latter shows but a small increase in mass on the former; so little in fact that it is well within the range of variation of successive subcultures on the same medium. But when the amount of moisture in growth of different ages is determined, it is found that the dried bacteria obtained from the 48-hour culture and expressed as a percentage of the moist growth, may be 1.5 to 2.5 times the percentage yielded by the 24-hour culture. Therefore it is very important that all measurements which are to be compared one with another should be made after approximately equal incubation.

The time interval we have chosen to incubate our cultures for the purpose of measuring growth is 24 hours. This choice was determined by the convenience it offered in the investigation of the growth yielded by successive subcultures on a given medium; although we use much younger cultures (14 to 16 hours) for virulence tests, because autolysis is then very much less marked than it is in older cultures, and presumably a greater proportion of the cocci are viable and there is less chance for interference by liberated endotoxin.

Even greater difficulties are encountered in the endeavour to obtain a standard inoculum for the plates in order to have really comparable measurements of growth. We are far from having succeeded in this; particularly when successive generations are studied.

### III. THE VARIATION IN GROWING POWER EXHIBITED BY MEDIA.

It is universally recognised that different kinds of media may give widely varying yields of growth, but perhaps it is not sufficiently realised, not only that different batches of any medium made by a described method give noticeably differing results, but that there is a considerable variation in yield of growth on any one batch.

The figures given in Table I, compiled from batches of media on which a sufficient number of observations have been made, are instructive.

The first point we wish to emphasise is that the yield of meningococcal growth per unit area, whether in terms of moist growth or dried cocci, varies enormously in different cultures on the same batch of medium and that these differences are usually not perceived by naked eye examination. It is for this reason that Murray (1924) stressed the point that it is not possible to establish a constant Minimal Lethal Dose for a strain of meningococcus in terms of agar slopes, Roux bottles, etc., as has often been attempted.

But still more important, from the point of view of the present paper, is the large difference between the figures representing the variation for any one batch of medium and the figures derived from observations of a number of batches representing the variation for the kind of medium. These variations are brought out particularly well when expressed as a percentage increase or

decrease on the arithmetical mean for the separate batches and for the kind of medium, and we claim that the divergence between the sets of figures demonstrates that it is extremely difficult to make two batches of medium exactly alike. We have taken a great deal of trouble in attempting to make media of uniform quality, but the above figures show that we have not succeeded to any great extent. Nevertheless, that we have not failed altogether is demonstrated by the figures representing the dried cocci as a percentage of the moist growth. It is clearly shown that in "Trypagar" and "EDB/N" the variations for the batch and those for the medium diverge widely; but on turning to "EDB/S" it is seen that there is a much closer correspondence between the two sets of figures. The importance of this point has been discussed already (Murray, 1924) and need not detain us here.

Table I.

Medium	Yield of moist growth per sq. cm. in mgms.				Yield of dried growth per sq. cm. in mgms.				Dry as % of moist growth			
	Maximum	Arithmetical Mean	Minimum	Maximum and Minimum as % increase or de- crease on Mean	Maximum	Arithmetical Mean	Minimum	Maximum and Minimum as % increase or de- crease on Mean	Maximum	Arithmetical Mean	Minimum	Maximum and Minimum as % increase or de- crease on Mean
Trypagar No. 3	1.75	1.45	1.20	+20.7 -17.2	—	—	—	—	—	—	—	—
5	1.68	1.63	1.55	+3.1 -4.9	0.329	0.304	0.273	+8.2 -10.2	22.0	19.9	17.6	+10.8 -11.3
6	1.40	1.29	1.15	+8.5 -10.9	0.243	0.223	0.175	+9.0 -21.5	18.1	16.8	14.7	+7.5 -12.5
3 batches of Trypagar taken together	1.75	1.43	1.15	+22.4 -19.6	0.329	0.258	0.175	+27.5 -32.2	22.0	19.0	14.7	+15.8 -26.3
EDB/N No. 36	3.11	2.73	2.27	+13.9 -16.9	0.524	0.448	0.380	+17.0 -15.2	16.9	16.2	15.5	+4.3 -4.3
37	3.40	2.85	2.06	+19.3 -16.9	0.562	0.488	0.413	+15.2 -15.4	20.1	17.3	15.8	+16.2 -8.7
48	2.77	2.63	2.44	+5.3 -7.2	0.500	0.437	0.388	+14.4 -11.2	19.0	17.3	15.1	+9.8 -12.7
63	2.54	2.12	1.79	+19.8 -15.6	0.488	0.415	0.355	+17.6 -14.5	22.2	19.6	18.0	+13.3 -8.2
72, 73	2.53	2.30	1.91	+10.0 -17.0	0.460	0.406	0.349	+13.3 -14.0	19.3	17.6	15.8	+9.7 -10.2
14 batches of EDB/N taken together	3.40	2.38	1.44	+42.9 -39.5	0.562	0.432	0.227	+30.1 -47.5	22.2	18.0	15.1	+23.3 -16.1
EDB/S No. 105	2.21	1.90	1.52	+16.3 -20.0	0.436	0.364*	0.300	+19.8 -17.6	19.9	19.3	17.9	+3.1 -7.3
110	1.74	1.63	1.54	+6.8 -5.5	0.338	0.335	0.333	+0.9 -0.6	21.6	20.3	19.4	+9.0 -4.4
5 batches of EDB/S taken together	2.51	1.83	1.19	+37.2 -35.0	0.546	0.369	0.296	+48.0 -19.8	21.9	19.9	17.9	+10.05 -10.05
EHD/V No. 196	3.8	3.2	2.6	+18.8 -18.8	0.67	0.51	0.43	+31.4 -15.7	17.7	16.1	14.8	+9.9 -8.1
198	4.0	3.1	2.3	+29.0 -26.0	0.75	0.52	0.40	+44.2 -23.1	18.9	16.8	15.1	+12.5 -10.1
202	3.9	2.8	2.2	+39.3 -21.4	0.60	0.45	0.36	+33.3 -20.0	16.8	16.2	15.8	+3.7 -2.5
209	2.7	2.1	2.0	+28.6 -4.8	0.48	0.35	0.30	+37.1 -14.3	17.5	16.4	15.2	+6.7 -7.3
4 batches of EHD/V taken together	4.0	2.8	2.0	+42.9 -28.6	0.75	0.45	0.30	+66.6 -33.3	18.9	16.3	14.8	+15.3 -9.2

It would appear that in obtaining this degree of success in making "*EDB/S*" medium, a proportion of the yield of growth per unit area had to be sacrificed and even were this necessary it would be worth while. Nevertheless, this does not entirely represent the truth, for further study of this type of medium has enabled us to make a medium, "*EDB/V*" or "*EHD/V*" from which we obtain a yield of growth even superior to "*EDB/N*" without losing the stability of the percentage yield of dried cocci characteristic of "*EDB/S*." Other experiments now in progress seem to promise still further improvements. We do not claim that the superiority of the latest form of our medium over those previously used is entirely due to any one factor, for it is exceedingly difficult to determine the effect of varying even one constituent because its influence is often closely bound up with the general balance of the remainder. In order to realise the result of an alteration it is frequently necessary to re-examine the whole question, by determining the optimal concentration of each constituent of the medium in relation to the primary alteration of the factor under examination. To do this very thoroughly would almost be a life's work because of the infinite number of possible combinations. We shall, however, attempt, in Sections IV and V, to give an indication of the influences of the separate constituents of the medium.

Here we would draw attention to the fact, that the average yield of growth on our *EHD/V* medium is really a very large mass for the meningococcus. The figures given for medium 209 in Tables I and II were obtained using a strain which normally gives smaller growth on any medium than any strain used to obtain the other figures in the tables.

We have shown that there is a very considerable variation in yield by different cultures on the same batch of medium. One aspect of this fact has been referred to as a periodic wave of growth by Murray (1924, p. 180), in its bearing upon the correct measurement of a dose of living cocci for injection into animals. We cannot, however, demonstrate a regular periodicity. In Table II we give the figures obtained in measuring the amount of growth yielded by media with successive subcultures at 24-hour intervals (= generations) of a strain of meningococcus. The plates were inoculated sufficiently heavily to try to be sure of confluent growth.

There is one point we wish to emphasise because it bears an important relation to the establishment of a Minimal Lethal Dose for living meningococcus cultures: that subsequent to the second generation on a medium, the yield of growth in successive subcultures at 24-hour intervals almost always exhibits more pronounced variation. The growth obtained in the first two generations may be described as being on the up-grade and when a culture is used for the inoculation of mice with purpose to determine the virulence of a strain, we consider it important to be reasonably sure that the growth is on the up-grade. For that reason we select the first, or more usually the second, generation for virulence tests. The effect of successive subculture, at intervals greater than 12 hours, on the killing power of a strain also makes it desirable

Table II.

Successive generations	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Modified Tryparag No. 27	—	1.46	1.90	1.61	1.41	2.06	1.51	1.70	5th generation growth not confluent									
Strain "Netley"	{	0.226	0.346	0.263	0.240	0.366	0.253	0.320										
% D	—	15.5	8.2	16.1	17.0	18.3	16.7	8.8										
EDB/N No. 36	{	2.97	3.11	2.47	2.53	2.65	3.10	2.27	6th gen. confluent growth on one plate only (scraped), the others discrete									
Strain "Netley"	{	0.480	0.520	0.384	0.415	0.433	0.524	0.380	colonies									
% D	—	16.1	16.7	15.5	16.4	16.3	16.9	15.5										
EDB/N No. 37	{	2.92	2.06	3.12	3.40	2.76	3rd gen. one plate confluent (scraped), one discrete colonies, and one no growth											
Strain "Netley"	{	0.460	0.413	0.662	0.556	0.449	16.3											
% D	—	15.7	20.1	18.0	16.4	16.3												
EDB/N No. 63	{	2.08	1.89	1.97	2.03	1.96	2.06	2.43	1.79	2.49	2.01	2.41	2.38	2.54	1.85	1.87	1.84	2.44
Strain "Netley"	{	0.420	0.356	0.386	0.376	0.436	0.413	0.464	0.358	0.485	0.406	0.437	0.488	0.455	0.363	0.375	0.355	0.484
% D	—	20.3	18.8	19.6	18.5	22.2	20.1	19.1	20.0	19.4	20.2	18.1	20.5	18.0	19.6	20.0	19.3	19.8
EDB/S No. 105	{	1.92	1.70	1.83	1.98	1.88	2.11	1.92	1.91	2.07	1.74	2.21	6th gen. all plates discrete colonies. 11th gen. no growth					
Strain "Netley"	{	0.353	0.318	0.327	0.371	0.351	0.404	0.373	0.380	0.407	0.344	0.436						
% D	—	18.4	18.7	17.9	18.8	18.7	19.2	19.5	19.9	19.7	19.8	19.7						
EHD/V No. 202	{	3.44	2.87	3.47	2.64	2.30	2.25											
Strain 28	{	0.546	0.423	0.546	0.422	0.381	0.356											
% D	15.9	14.7	15.8	16.0	16.5	—	16.1	15.6	16.4	16.8								
EHD/V No. 209	{	2.04	2.00	2.17	2.01	2.08	6th gen. all plates discrete colonies. 11th gen. no growth											
Strain Pettigrew	{	0.316	0.305	0.365	0.306	0.361	16.4											
% D	15.4	15.4	16.8	15.2	17.4	—												

M = Moist weight in mgms. per sq. cm. D = Dried weight in mgms. per sq. cm. % D = Dried growth as a % of moist growth.



to use as early a generation as possible; but this question will be raised again in Section VI (Table XI).

This question of marked fluctuation in the yield of growth on a given medium is one which deserves closer consideration than we have been able to give to it, and, probably, it involves physiological processes which are important to the micro-organism.

It is possible that a sufficiently even and regular growth would result if the viable state of the inoculum used for the successive generations was reasonably constant, and the fact that this condition is very difficult to realise suggests very strongly that the variation in yield of growth, on a given batch of medium, is due to the state of the organism rather than to the medium. For some time past we have made it a rule to take only discrete colonies growing at the margin of an inoculated area as the inoculum for the succeeding generation, but, although this is an improvement, it does not interfere appreciably with the usual fluctuation or even the occasional complete death of a culture.

#### IV. AN ATTEMPTED STANDARDISATION OF A MEDIUM.

Having considered the variation in yield exhibited by media, the ground is cleared for the examination of the various phases of preparation of the medium and to discuss their relative values. It is unnecessary to recapitulate what is known of the substances found in media which successfully grow parasitic bacteria, since we have nothing to add to the broad classification of them to be found in books dealing with the nutrition of living organisms. That with which we are more intimately concerned is the manipulation of the raw materials ordinarily used for media making and we shall show that this subject still provides a wide field for research of which we have barely touched the fringe.

Broadly speaking the type of medium we are considering consists of a watery extract of fresh meat, to which is added the products of tryptic digestion of meat, inorganic salts and accessory growth substances in the form of body fluids and exudates or extracts of animal or plant tissues. This complex mixture is usually held in a jelly of agar and clarified by the coagulation in it of some albuminous substance. The reaction is adjusted to a desired degree of acidity or basicity and it is then sterilised by raising it to the thermal death-point of living matter.

It is with the details of these various manipulations that we wish to deal in this section.

##### (a) *The meat extract.*

The tissue we confine ourselves to for the present is bullocks' heart muscle and we consider it a matter of primary importance to use only freshly killed meat: we refuse any which has been dead for more than 24 hours. This is freed of fat and the larger vessels, not too finely minced and extracted with one litre of tap-water to each 500 gms. of mince at between 70° and 75° C. for three

hours. After that the temperature is raised rapidly to 100° C.; a large amount of protein is thus coagulated and this is immediately preceded by an evolution of gas (93° to 95° C.). The water lost during heating is made up by adding distilled water to the original weight of the mixture, which is then filtered. The filtrate is bright and perfectly clear and is of pinkish-yellow colour. We have made numerous observations on the weight of the meat before and after extracting and pressing and also on the volume of the fluid yielded as a finished product and we find that between 50 and 60 per cent. by weight of the meat is yielded up to the extract. The filtered extract is bottled and sterilised at 120° C. for 20 minutes when a further coagulation takes place and the pinkish tinge is lost. During the last six months we have made our extracts with *distilled* water and on autoclaving the resulting filtrate it retains more of the ruddy tinge and there appears to be considerably less secondary coagulation. We believe the substitution of distilled water for tap water to be a marked improvement for additional reasons to be discussed in the subsection dealing with the influence of certain inorganic salts.

The constancy of the product obtained in this way is shown by the figures given in Table III.

Table III.

Extract No.	Total solids gms. in 100 c.c.	Ash gms. in 100 c.c.	Total Cl <sub>2</sub> gms. in 100 c.c.	Total N <sub>2</sub> gms. in 100 c.c.	Sørensen figure (see text) in c.c. N/10 NaOH on 10 c.c.	c.c. N/100 NaOH required to raise reaction of 1.0 c.c. from pH 7.0 to pH 8.0 to phenol red	Notes
40	1.56	0.41	0.035	0.166	1.7	0.6	Tap water
132	1.76	0.33	0.035	0.136	1.5	0.5	"
134	1.45	0.36	0.035	0.144	1.7	0.7	Distilled water
158	1.52	0.36	0.053	0.150	2.1	0.6	"
163	1.58	0.41	0.035	0.158	1.8	0.7	"
199	1.40	0.44	0.035	0.140	1.8	0.9	"

The method we have elaborated for filtering the meat extract deserves mention because it is very much more rapid than filtration through paper and is quite as efficient.

A sheet of fine butter-muslin is pinned on to a wooden frame in such a way that when the frame is rested in a large funnel the bag formed by the muslin does not touch the funnel. The extract and meat are stirred up and when the main part of the coarse meat has settled, leaving the fine coagulum still in suspension, it is rapidly poured into the wetted muslin bag so as to fill it. The fine coagulum settles uniformly on to the muslin and forms the actual filter, the excess of coarse meat is scooped out with a cup and returned to the can and the filter is then left undisturbed until the filtrate runs clear; this takes less than five minutes. Meanwhile the vessel containing the extract has stood undisturbed and the meat has settled together with the fine coagulum. The supernatant fluid, together with the first runnings, is now decanted into the centre of the filter, taking care not to fill above the filter-bed on the muslin and without unduly disturbing either the filter-bed or the meat in the can.

When the bulk of the fluid has been poured off from the meat, the can is balanced on the wooden frame at an angle to allow the remaining fluid to drain away into the filter, leaving the meat behind with most of the fine coagulum entangled in it. In this way we are able to save hours of time compared with filtration through paper. Starting with 1200 gms. of meat and 2400 c.c. of water, 2000 c.c. of perfectly clear filtrate was obtained in 15 minutes, and 2650 c.c. in less than 50 minutes without pressing the meat residue.

(b) *The digest.*

At the commencement of the present investigations we selected "Trypagar" (Gordon, Hine and Flack, 1916) as our routine medium, but we soon observed that it was extremely difficult, if not impossible, to make two batches sufficiently alike; the variation we observed is illustrated in Table I. In a previous investigation (unpublished) it was observed that the production of toxin by dysentery bacilli varied with the batch of medium and the medium then used had one point in common with "Trypagar" in that both are made with Douglas broth as a basis. We thought that in making Douglas broth the variable which was least under our control was the degree of digestion undergone by the meat and we decided to add a digest to a meat extract in such a proportion that every batch of medium would have an identical concentration of amino-acids, as determined by titration in the presence of formaldehyde. The amino-acid concentration in different digests varied considerably, but in the finished medium the increment of amino-acids in 10 c.c. of medium, due to added digest, neutralised 2.5 c.c.  $N/10$  NaOH (= approximately an  $N/40$  solution). This figure was determined by experiment to give maximum growth.

In making the digest no strict precautions were taken at first to exclude contamination and the presence of micro-organisms was easily demonstrated, although growth during the time allowed was slight in the high concentration of the products of tryptic digestion which accumulated very rapidly, but anaerobes were usually sufficiently numerous to cause an unpleasant smell.

The medium made in this way we call "*EDB/N*" (*E* = extract, *D* = digest, *B* = Blood, and *N* = non-sterile digest) and the variation it exhibited is shown in Table I. These figures show a much improved yield of growth when compared with "Trypagar," but in other respects this medium has no particular claim to superiority. The growth when scraped off in mass had a curious flesh-pink tinge which was not seen in growth from "Trypagar," but which obtains with all our media of the *ED* type. Although we used this medium extensively and the growth it yielded per unit area was satisfactory, we shall not consider it in detail because it would unduly burden this paper to describe every step in the investigation. We propose to consider as briefly as possible only the chief points which led to the development of the technique we have provisionally adopted.

At this stage we thought that some degree of control over the concentration

of amino-acids in the medium was the all-important factor. However, in attempting to determine the virulence of strains of meningococcus grown on this medium, we obtained ample evidence of the inconstancy which has been so commonly emphasised as characteristic of that organism. But it must be remembered that at that time we had not recognised the more important details of technique which it is essential to observe in order to determine the virulence of a culture and to ensure the successful repetition of an experiment. We were contented for the time being with the idea that maximal growth indicated a good medium and assumed that such a culture was healthy and probably possessed the physiological characters of the disease-producing meningococcus.

Nevertheless we were uneasy about what might be the influence of the slight but mixed infection of the digest on the efficiency of our medium and so proceeded to digest the meat with such strict precautions that contamination was completely avoided. Such sterile digests were used to make the medium called "*EDB/S*" (*S* = sterile) in Table I, maintaining the same increment of amino-acids. At first we were greatly disappointed with this medium because of the poor yield of growth per unit area compared with *EDB/N*, but an examination of the properties of the growth caused us to abandon *EDB/N* as a bad medium; but we have learned since that it was the general adjustment of the medium which was at fault and not the digest (see Section V, Table VIII). Two of our reasons are illustrated in Table I: firstly, the moisture represented as a percentage of the living growth is much less variable than the media used up to this time; secondly, the yield of dried growth per unit area is not greatly inferior to *EDB/N*. A third reason, to which we attached considerable importance, must be stated briefly here although it will be dealt with in greater detail later. Batches of both *EDB/N* and *EDB/S* then in use were inoculated from the same culture of a given strain of meningococcus and it was found that the growth from the former failed to kill mice and rats whereas the growth from the latter killed quite regularly. This result was confirmed with certain other batches of these media. Although the interpretation of these results is a matter of difficulty, it appeared to be evident to us that there was a difference in the physiological state of the bacteria as grown on these media and that the mass of growth yielded by a medium was less important than the killing power of a culture.

At this time the general treatment, the relative concentrations of meat, water and trypsin, and also the reaction and time of digestion were as near as we could make them alike for the different digests, but the contaminated digests always showed a very much higher concentration of "amino-acids," in terms of c.c. *N/10* NaOH in the presence of formaldehyde, than the sterile digests did. This is probably due to an enterokinase-like substance supplied by the contaminating bacteria (see Richet and Richet, 1921, p. 1060).

However, since the amino-acid increment due to digest in the finished medium was the same in both *EDB/N* and *EDB/S*, the property it is desirable

to control is definitely independent of the general amino-acid figure, determined by titration in the presence of formaldehyde. This is indicated by both growth and killing power (see Section V).

In order to investigate this matter further numerous experiments were made to determine approximately the conditions necessary to allow the sterile digests to contain an equally high concentration of amino-acids as the contaminated digests, and guided by the results of this investigation we have adopted the following method of making our digest:

Immediately after the extract has been drained away the residual meat mince is suspended in as many litres of  $N/100$  HCl as there were kilos of original fresh meat before extraction, and put into a flask fitted up so that samples can be withdrawn and sterile fluids added when required. This is then autoclaved for 30 minutes at  $130^{\circ}$  C. to ensure sterility; it is advisable to heat large volumes of material to  $100^{\circ}$  C. in a water-bath or steamer before putting them into the autoclave. When cooled sufficiently the flask is placed in an incubator at  $37^{\circ}$  C. and left overnight in order to make certain that the large bulk of material reaches the same temperature throughout and to test for sterility.

The pancreatic extract is sterilised by filtering it through a Pasteur-Chamberland candle "F" and is added in the proportion of 20 c.c. to each litre of  $N/100$  HCl used. It is essential to add the trypsin to the acid in order that it may be activated (see Richet and Richet, 1921, p. 1060). We have found by experiment that the amount of digestion which takes place in a given time, other things being equal, is very much greater when the pancreatic extract is added to an acid substrate than when it is added directly to one of  $pH$  8.0 (the region of optimal reaction for tryptic digestion).

We find that trypsin can be activated quite as well by treating it with  $N/100$  HCl before adding it to the meat suspended in an alkaline medium. Adding it to an alkaline medium containing 0.5 per cent.  $CaCl_2$  (see Richet and Richet, 1921, p. 1060) is also effective.

The routine we have adopted is to add the trypsin to the meat suspended in  $N/100$  HCl and after 5 hours' contact to adjust the reaction to  $pH$  8.0 as described subsequently and allow digestion to proceed at  $37^{\circ}$  C. Originally the reaction was adjusted by adding  $10N$  NaOH, and sufficient was added at once to maintain the alkalinity over the desired time; this was found to be 15 c.c. per litre. As this raised the alkalinity at the commencement slightly more than is desirable we now add the equivalent quantity of  $Na_2CO_3$  (= 0.8 per cent. anhydrous  $Na_2CO_3$ ). We have not found our trouble sufficiently rewarded when we added the necessary alkali at short intervals during the course of digestion, and it increases the risk of contamination. Digestion is allowed to proceed for two to three weeks and during that time somewhere in the region of 10 per cent. of the meat is digested.

That the degree of digestion is sufficient for our purpose is indicated by titrating the amino-acids in the presence of neutralised formalin using phenol-

phthalein as indicator and expressing the result in c.c.  $N/10$  NaOH required to neutralise the amino-acids in 10 c.c. of the filtered digest: this we call its Sørensen figure and for reasons to be discussed later we require our digest to have a Sørensen figure not less than 20. The digest is usually very dark coloured and when it is undiluted it is extremely difficult to see the end-point of the indicator. Therefore, in making our titration, 5.0 c.c. of the digest is made up to 25.0 c.c. with distilled water, boiled and rapidly cooled and titrated to the first faint change of the phenolphthalein with  $N/10$  NaOH; a similar sample is titrated to the same point and kept as the control colour; 5.0 c.c. of freshly neutralised formalin (40 per cent.  $C.H_2O$ ) is now added and the mixture titrated to match the control. This second increment of  $N/10$  NaOH represents the Sørensen figure for 5 c.c. of the digest. We always check the result by adding excess of alkali and allow the mixture to stand for ten minutes, then titrate back with  $N/10$  HCl. We have found repeatedly that amounts of formalin up to 4 c.c. give an increasing Sørensen figure and that amounts over 5 c.c. do not alter the result. We usually find it necessary to redistil commercial formalin to obtain a satisfactory solution for this titration, otherwise it discolours on neutralisation.

(c) *The concentration of amino-acids.*

We stated above that whatever the concentration of amino-acids in the digest might be, we only added sufficient to increase the Sørensen figure of the extract by a definite amount which had been determined by experiment. This procedure served a useful purpose by indicating two important points: (1) That the yield of growth per unit area is not dependent on the general concentration of amino-acids in the medium as represented by the Sørensen figure. This point is amply demonstrated by the figures given in Table I where the media described as *EDB/N* and *EDB/S* have the same Sørensen figure and only differ in that the latter contains an actually greater percentage of digest. (2) That, as has been mentioned already, and will be discussed in detail later, the killing power of a given strain of meningococcus has been observed to vary constantly with the medium on which it is grown. In the instance already mentioned the two media had the same Sørensen figure.

Since we are satisfied that a constant concentration of amino-acids in the finished medium, as expressed by the Sørensen figure, does not contribute to the stabilisation of the culture either in respect of yield of growth or killing power and as sufficient evidence to support this conclusion is to be found in Table I and Section V of this paper, no useful purpose would be served by detailing experiments.

It has been our experience that the amount of substances in the digest which are essential to produce the most efficient medium bear some relation directly proportional to the degree of digestion indicated by the Sørensen figure of the digest. We do not consider digestion to have proceeded sufficiently far for our purpose until a Sørensen figure of not less than 20 has been reached.

*(d) The added inorganic salts.*

Among the characters used for the recognition of meningococcus colonies in cultures from the nasopharynx are the consistency of the growth and the ease with which it emulsifies. It is said that it "picks up like paint"; it ought to be soft and not stick to the medium, neither ought it string out like mucilage nor be friable; and it ought to emulsify readily and evenly.

In working with pure meningococcal cultures isolated from cerebro-spinal fluids and grown on the many varying media we have made for experimental purposes, we have frequently obtained growths which exhibit those very characters the typical meningococcus is supposed not to possess. Quite commonly the growth is very slimy and difficult to remove from the surface of agar media and is very obstinate to emulsify in physiological saline. When picked up it strings out into an almost elastic thread and maintains that character when placed in saline, giving a very imperfect emulsion even after prolonged shaking. On the other hand, the growth may pick up quite readily but have a matt, granular appearance and when scraped into a heap the surface appears to be dry and wrinkled. This second type of growth gives a flocculent emulsion which very rapidly settles out of suspension. These are extremes which might be called the "Sticky" and "Granular" and can be produced by any given strain on two different media and be associated in each case with a very good yield per unit area. Somewhere between the two occurs the "characteristic" smooth, moist, flesh-coloured growth, which emulsifies easily and smoothly.

Microscopically no morphological distinction can be drawn between the cocci constituting the mass of growth exhibiting these various characters.

Possibly these observations explain certain disagreements in the literature.

Before entering into the details of our observations, as far as we have carried them, it is as well to draw attention to the remark of Nicolle, Debains and Jouan (1918, p. 151) that: "If it is desired thoroughly to know the macroscopical appearance of meningococci, it is necessary to examine them in a mass of several grammes."

There are two influences exercised by these physical characters exhibited by meningococcal cultures: The first is their influence upon the measurement of the growing power of a medium and on this the "granular" type of growth has no effect, but the "sticky" kind is difficult to scrape up cleanly and without the risk of picking up pieces of medium or unduly squeezing moisture out of the agar. Although it only introduces a marked error in the case of extreme stickiness, it has the disadvantage of being difficult to manipulate. The second influence depends upon the imperfect emulsion they produce and has more profound effects upon general experimental work, such as inoculating animals or measuring quantities by a dilution method, etc., and this is particularly marked in the case of the rapidly sedimenting emulsion resulting from the "granular" kind of growth.

We first endeavoured to discover whether these physical characters of the growth were associated in any way with the frequently observed capsulated appearance of the cocci and the virulence of the culture, but we failed to find any such association. Since the virulence of a culture remained unaffected by them it was desirable to eliminate the extreme variations, which were a source of trouble in the general experimental work and we turned to the effect of varying the concentration of salts in the media. Although we do not pretend to have made a complete investigation of the influence of salts, we have obtained certain interesting results and to some extent we have succeeded in eliminating the extreme slimy and granular types of growth.

All the experiments now to be considered were made with media of the *EDB/S* type.

In the first place there were occasions when we accidentally omitted to add NaCl and  $\text{CaCl}_2$  to the medium used for general work and confirmed their absence by titration subsequent to absolute failure to obtain growth on those batches, although other batches made with the same materials were satisfactory. These defective media gave the usual growth when we added the salts in the concentrations we then used (0.5 per cent. NaCl, 0.0125 per cent.  $\text{CaCl}_2$ ). At this time we made our extracts with tap water. On one occasion we reserved a portion of the medium which gave no growth without and good growth with salts, added 0.0125 per cent.  $\text{CaCl}_2$  and varied the NaCl concentration with the following results:

- (1) 0.25 per cent. NaCl gave growth which was not sticky and scraped well.
- (2) 0.50 per cent. NaCl gave growth which was moderately sticky but scraped well.
- (3) 0.75 per cent. NaCl gave growth which was sticky and scraped badly.
- (4) 1.00 per cent. NaCl gave growth which was very sticky and would not scrape at all.

Medium (3) gave very nearly half the yield per sq. cm. given by media (1) and (2) which were approximately equal. This very definitely demonstrates that sticky growth can be due to a too high concentration of NaCl. Subsequent experiments showed that in the presence of 0.0125 per cent.  $\text{CaCl}_2$ , when the extract has been made with tap water, the range of concentration of NaCl varying between 0.1 per cent. and 0.3 per cent. gave smooth moist growth and that stickiness commenced to appear with 0.4 per cent. and increased with higher concentrations of this salt. No lower concentration than 0.1 per cent. was tried at this time.

Without entering deeply into the question, we selected 0.25 per cent. as the concentration of NaCl giving the most desirable form of growth for our purposes and then proceeded to test the effect of varying the concentration of other salts.

Media made with tap water extracts and containing 0.25 per cent. NaCl without additional  $\text{CaCl}_2$  invariably gave sticky growth and the following



two experiments (Table IV) are instructive in showing that stickiness can be sufficiently inhibited by adding an appropriate amount of  $\text{CaCl}_2$  and that an excess only increases this defect in the presence of  $\text{NaCl}$ .

Table IV.

Experiment	Medium No.	% NaCl	% $\text{CaCl}_2$	Character of growth	Regrowth in 9 hours over area scraped in morning
I	1	0.250	0	Sticky, scrapes badly, emulsifies easily	—
	2	0	0.0125	Smooth, scrapes well, emulsifies easily	—
	3	0.250	0.0050	Sticky, scrapes well, emulsifies easily	—
	4	0.250	0.0200	Slightly sticky, scrapes well, slightly stringy emulsion	—
	5	0.250	0.0400	Sticky, scrapes fairly well, stringy emulsion	—
II	A	0.250	0	Very sticky, scrapes badly	Grown well
	B	0	0.010	Smooth, scrapes well	Not grown
	C	0.250	0.005	Sticky, scrapes fairly well	Grown well
	D	0.250	0.010	Smooth, scrapes well	Not grown

It appears from these experiments that in the presence of 0.25 per cent.  $\text{NaCl}$  the concentration of  $\text{CaCl}_2$  required to give smooth moist growth is between 0.01 and 0.02 per cent. and probably nearer the smaller figure. A result which we have confirmed using media made with distilled water extracts.

At the time of doing Exp. II (Table IV) we had become interested in the question of regrowth of the meningococcus over the area from which the growth had been scraped cleanly and in the appearance of secondary colonies superimposed on previously existing growth. The latter phenomenon had occurred very rarely and usually only after prolonged incubation on *EDB/N* media and was the rule with *EDB/S* media, when such media exhibited a difference in virulence in the cultures. We were inclined on that account to stress the importance of sterile digests (see Section VI) and it is interesting, therefore, to notice the inhibition of regrowth by 0.01 per cent.  $\text{CaCl}_2$  in Table IV.

Since we attach importance to regrowth as a criterion of a good medium, this effect of  $\text{CaCl}_2$  caused us to try the effect of adding  $\text{KCl}$  to the medium; because of the high concentration of potassium salts described in actively growing malignant growths and the high concentration of those salts in egg-yolk; egg medium being one on which the meningococcus lives a very considerable time.

To an *EDB/S* medium containing 0.25 per cent.  $\text{NaCl}$  and 0.01 per cent.  $\text{CaCl}_2$  we added  $\text{KCl}$  in varying proportions with the following results:

- (1) No  $\text{KCl}$  gave smooth growth and very slight regrowth.
- (2) 0.01 per cent.  $\text{KCl}$  gave slightly granular growth and very pronounced regrowth.
- (3) 0.015 per cent.  $\text{KCl}$  gave markedly granular growth and marked regrowth.
- (4) 0.02 per cent.  $\text{KCl}$  gave very pronounced granular growth and slight regrowth.

The regrowth in this experiment was noted after 24 hours' re-incubation,

while in the  $\text{CaCl}_2$  experiment (Table IV) it was noted after only 9 hours' re-incubation. This experiment is interesting not only because it suggests that the potassium salt has some influence on the regrowth of the culture over an area upon which it has already grown, but because the salt also appears to influence the production of the granular type of growth when both sodium and calcium salts are present. Further, when the  $\text{NaCl}$  and  $\text{CaCl}_2$  are present in the proportion named above the optimal concentration of  $\text{KCl}$  is identical for both regrowth and the least granular type of growth.

In another experiment the medium was made with distilled water extract and the salts were added in varying proportions, but in no medium were more than two salts present. As far as possible all the other conditions were maintained alike for the different media and the results obtained are shown in Table V.

Table V.

Medium No.	$\text{NaCl}$ %	$\text{CaCl}_2$ %	$\text{KCl}$ %	Mean weight in mgms. of dried growth per sq. cm.	Mean dry growth as a % of the mean moist growth	Physical character of growth	Regrowth 24 hours after scraping
1	0	0	0	0.525	17.1	Smooth	None
2	0.25	0	0	0.373	18.7	Very sticky	"
3	0.25	0.005	0	0.375	19.4	Sticky	Very slight
4	0.25	0.01	0	0.563	19.8	Smooth	"
5	0.25	0.02	0	0.445	19.6	"	"
6	0.25	0	0.01	0.511	19.3	Very sticky	Fair
7	0.25	0	0.02	0.431	19.7	"	Very good
8	0.25	0	0.04	0.448	19.0	"	"

There are two points to be noted in comparing this with other experiments previously described: firstly, that the media were made with a distilled water extract; and secondly, that freshly laked blood was added as the accessory growth factor, which, as will be shown later, in common with fresh ascites fluid stimulates growth under otherwise unfavourable conditions, and probably accounts for growth being obtained on medium No. 1. This experiment suggests:

(a) That the addition of  $\text{NaCl}$  causes stickiness which is counteracted by an optimal proportion of  $\text{CaCl}_2$  and which is enhanced by adding  $\text{KCl}$  without  $\text{CaCl}_2$ .

(b) That the addition of  $\text{KCl}$  stimulates regrowth over the area from which growth has once been removed.

(c) That  $\text{KCl}$  in the presence of  $\text{NaCl}$  and the absence of  $\text{CaCl}_2$  does not cause the growth to be granular.

We have not followed the question of the influence of inorganic salts as far as we would have liked, because it was not the primary object of our quest. We were mainly concerned with an attempt to render the conditions of growth such that the extremes of "Sticky" and "Granular" growth did not appear; for no other reason than that those extremes made our work more than ordinarily difficult. To some degree we have been successful, for, since adopting 0.25 per cent.  $\text{NaCl}$ , 0.01 per cent.  $\text{CaCl}_2$  and 0.01 to 0.02 per cent.  $\text{KCl}$  as the

concentration of those salts to be added to a medium made with distilled water extract, we have not experienced extremes of sticky or granular growth in other than experimental media to which excessively high concentrations of digest have been added.

Nevertheless, our investigation does not supply a complete explanation, for we possess one strain of meningococcus which is habitually slightly sticky on our ordinary media, though never unmanageably so and we shall show that there are other influences than inorganic salts tending to cause stickiness.

It is expedient to be aware of the concentration of NaCl in the digest within reasonable limits. With all media made from one particular digest we were troubled with sticky growth, until we checked the concentration of NaCl it contained as the result of neutralising the alkali which had been added in the course of digestion. On taking this into account and adding proportionally less NaCl to the finished medium, the growth obtained was of the desired consistency.

One other point deserves mention: the growth may be smooth and satisfactory when 12 to 16 hours old and quite noticeably sticky at 24 hours. In any case stickiness increases with age.

The killing power of the various strains used in these experiments was known and the growth yielded by these media, with varying concentrations of salts, gave no indication of variation in "virulence" for mice.

(e) *The reaction.*

It would be misleading were we to state that the reaction of our medium is adjusted to a definite  $pH$ , since we have not taken into account the salt and protein errors and the titration has not been done at a constant temperature. At present we are content to adjust the reaction by a standard method whereby we do obtain fairly constant results, because our titrations have but slight variations which are well within the relatively wide range of tolerance of the bacteria to changes in the reaction of their environment and because comparatively large quantities of alkali or acid are necessary to produce a marked change in the reaction of the medium. The colorimetric method is employed, with phenol red as the indicator and we match our medium to standard Sørensen solutions of phosphates, superimposing a blank to compensate for the natural colour of the medium.

The medium is heated in a water-bath to melt it and drive off the  $CO_2$  and 1 c.c. in a cordite tube is diluted with 4 c.c. of boiling distilled water. Two such tubes are prepared and to one of them the indicator is added in the same concentration as it is added to the tubes of Sørensen buffer solutions; the other tube is kept as the colour control. The diluted medium is made to match the buffer solution having a  $pH = 7.2$  and then it is boiled and cooled rapidly under the tap without shaking, before the final reading is made.

After the calculated amount of NaOH has been added to the bulk of the medium, we are careful to take a sample to check the reaction. Agar media

which have been titrated when diluted to prevent too rapid setting and to the bulk of which the calculated amount of alkali has been added, frequently are considerably on the acid side of the desired reaction. We have not investigated this point, nor do we offer any explanation, but we suggest that the omission to check the final reaction of the bulk of the medium has led to the statement having been made that ordinary media become increasingly acid with autoclaving. We have frequently autoclaved our finished media four and five times without the reaction changing in the process. On the other hand, if the desired reaction of the medium is more alkaline than that we require, then there is certainly a fall in the *pH* owing to the precipitation of phosphates, but it is not necessary to autoclave it in order to observe this change. When we have previously partially removed the phosphates from our media, we have usually found that the calculated quantity of alkali is sufficient to adjust the reaction to the desired *pH*. Liquid media (broth) do not behave in this peculiar way in our experience and the calculated quantity of alkali required to adjust the reaction to "*pH* 7.2" has always been correct, but, besides being free from agar, these media have not been diluted for purposes of titration.

The reaction we have selected is that which we found by experiment to yield maximum growth per unit area and it is certainly rather more acid than the optimal reaction generally claimed for the meningococcus.

(f) *The clarification.*

A perfectly transparent agar medium is so much a convenience as almost to be a necessity and is always preferable to another equally good in all other respects but this. When the extract and digest are clear to start with, there is little more needed to render the finished medium sufficiently transparent for almost all purposes, since the haziness due to the agar is but slight. But owing to the extract and digest being markedly acid the necessary adjustment of the reaction occasions a flocculent precipitate of phosphates to form, which if distributed through the medium interferes with its transparency. Although this precipitate readily deposits and the clear medium can be decanted off, an economy of time and medium can be effected by first filtering through ordinary surgical lint, which holds back the bulk of the precipitate as well as all the fine dirt in the agar. Were it not essential to add accessory growth factors to obtain cultures of freshly isolated or virulent strains of meningococcus, there would be no need to filter the medium at all. For a considerable time we met this need by adding freshly drawn horse blood which was coagulated by heat in the presence of the agar; this coagulum was removed most satisfactorily by filtration through lint, the medium held in the lint and clot was wrung out by hand, and the finished product was glass clear. The removal of this fine coagulum by filtration through paper is an extremely slow process and usually results in the loss of a considerable quantity of the medium.

Later, when freshly drawn horse blood was no longer available, we evolved

a process whereby the accessory growth factors are derived from the heart muscle used for making extracts and digests. The method will be described presently, but it is necessary to state here that an extremely fine coagulum is produced which is not removed easily by filtration through lint. However, we have found it to be more easily removed if it is produced and filtered in the presence of the glutinous phosphate precipitate, with which it seems to become entangled. We obtain this condition by adjusting the reaction of the medium to match the Sørensen solution of pH 7.6 (in the manner already described) before coagulation takes place. At this degree of alkalinity a copious precipitate of phosphates is produced which facilitates the removal of the fine protein coagulum by filtration through lint. The production of the phosphate precipitate reduces the alkalinity of the medium to some extent; so that the reaction now is usually that which we require for our finished product, namely pH 7.2. The filtrate obtained in this way is quite sufficiently clear, but usually a slight haziness remains; this can be removed by filtration through paper or silted up lint if desired, but if some specially clear medium is required it is easily obtained by decanting the top layer which has cleared itself by sedimentation. That only a fraction of the phosphates in the medium is removed by this process is demonstrated by making the medium much more alkaline subsequently (pH 8.0 or 9.0), when a heavy precipitate is produced immediately.

But a word of warning is necessary since we have observed, when we have purposely poured into a plate a sample of medium containing a copious precipitate of phosphates and other matter, in such a way that the precipitate is not distributed evenly, that then the growth of meningococcus is much thicker over the dense precipitate than it is over the clear portions of the plate. In fact, when the accessory growth factors are derived from the heart muscle, there is no doubt that we get better results when the medium is slightly cloudy than we do when it is glass clear. When, however, the accessory growth factors are obtained by coagulating blood in the medium with heat, perfectly cleared medium is quite as efficient as that which is cloudy.

(g) *The sterilisation.*

We have not been able to detect any alteration in the finished medium, nor in the individual ingredients after repeated autoclaving at 120° C. for 20 minutes, as tested by the yield of growth and the killing power of the meningococcus. When the bulk of material to be sterilised in this way is large, we take the precaution of raising it to 100° C. in a water-bath or steamer before autoclaving it, otherwise the desired temperature is not reached and sterilisation is incomplete. To test the temperature to which the material has been subjected we use pure chemical compounds of known melting point, enclosed in sealed glass tubes.

The statement has frequently been made, that, subsequent to autoclaving a medium, the reaction is more acid than it was to start with. We are em-

phatically of the opinion that this is not the case with media which are not markedly alkaline and do not contain sugars. There are two conditions which we consider explain such observations, but we do not know to what degree they are interdependent: firstly, that the calculated quantity of alkali has been added to the bulk of the medium and the reaction has not been checked prior to autoclaving; secondly, that the formation of a copious precipitate of phosphates has separated in a markedly alkaline medium and in so doing reduced the alkalinity. In any case the second eventuality ought to be safeguarded against by resorting to the obvious expedient of checking the reaction before sterilisation, and, if necessary, removing the excess of phosphates.

We have not been able to discover that repeated autoclaving produces any deterioration of the stimulus to growth due to the accessory growth factors which are essential to successful cultivation of the meningococcus.

#### (h) *The accessory growth factors*

It is well known that in primary culture the meningococcus requires these accessory substances and probably this is the principal reason why it is universally understood that special media are necessary to grow it. The absolute need for special provision of these accessory factors decreases with the time a given strain has been kept in culture and the meningococcus can readily be educated to grow on "ordinary media," as is a common practice in laboratories. But two outstanding features of the life of this organism *in vitro* definitely prevents any application of this practice to the work we are doing at present: firstly, freshly isolated strains or those which have been raised in virulence will not grow readily on ordinary media; and, secondly, the repeated subculture necessary to accustom the organism to such media immediately deprives the cultures of their power to kill mice. Thus it is essential, apart from any other properties the medium may possess, to provide the required accessory growth factors.

It is not our purpose to consider these substances with any intention to classify them, but the manner in which we used the fluids containing them falls naturally into two categories:

(1) Their addition to the medium immediately before use and after sterilisation.

(2) Their addition as an integral part of the preparation of the medium before sterilisation.

The first method requires that the substances be added to the otherwise finished medium with strict sterile precautions and at the same time exercising minute supervision over other conditions, in order that the supposedly delicate accessory growth factors are not subjected to treatment which either destroys or removes them: such as autoclaving or adsorption by large surfaces provided by filter paper or finely divided precipitates. The second method allows of the accessory growth substances, in the presence of the other constituents of the medium, to be subjected to the usual processes of sterilisation, filtration, etc., without interfering with their function. Obviously this has great advantages.

The substances we have tried by the first method are: ascites fluid, horse serum, laked horse blood, formol serum and extracts of red corpuscles. The substances we have tried by the second method are: Gordon's extract of pea-flour, freshly drawn horse blood, extracts of red corpuscles, formol serum and our own method of using extracts of heart muscle.

So much for the categoric treatment of the subject and we shall now consider each of the individual substances by their effect upon growth.

*Ascites fluid*, when freshly drawn and added immediately before use to agar media which have been cooled to 45° C., is undoubtedly superior to any other substance we have tried. In our experience, it enables media which are otherwise absolutely unsuitable for meningococcal growth to give a remarkable yield per unit area. Nevertheless, in addition to the difficulty of maintaining an adequate supply and contrary to the invariable statement of the case, ascites fluid does not keep well exposed to the air in vessels plugged with wool. We have not tried hermetically sealed vessels. Using the same batch of medium, or batches made as nearly alike as we know how, to which was added 5 per cent. of ascites fluid immediately before pouring the plates, we obtained profuse, smooth, creamy growth when the fluid was freshly drawn. As the fluid aged (10 days) the growth tended to become sticky, until, at last, it became unmanageable, reaching such a degree of stickiness that it could not be removed from the surface of the agar (30 days). As the sliminess of the growth increased the yield appeared to become less. We were inclined, at first, to blame every process and ingredient used in making the medium except the ascites fluid, and we spent a considerable time investigating the effect of various alterations, without improving matters until we examined the ascites fluid. Ascites fluid which had been kept in vaccine bottles with wool plugs for 30 days was very alkaline, well beyond pH 8.0; when it was 50 days old, 5 c.c. of it required 0.7 c.c. N/10 HCl to adjust its reaction to pH 7.2, in spite of the fact that the fluid appears to be comparatively slightly buffered. When the reaction of 5 c.c. of this fluid containing phenol red was adjusted to pH 7.2 and left standing in a boro-silicate glass tube for 15 hours exposed to the air, the upper layer of the fluid became very much more alkaline than the lower layer.

A hundred c.c. of ascites fluid was placed in a wool-plugged flask and the reaction was adjusted to pH 7.2 with full sterile precautions; it was then left in a cool dark cupboard for 6 days, when the reaction was found to be pH 8.0 and 5 c.c. required 0.3 c.c. N/10 HCl to restore the reaction to pH 7.2. Every precaution was taken to reduce the absorption of alkali from the glass. Two litres of the same fluid were stored in the cupboard in a Winchester quart bottle with a rubber bung and only exposed to the air through a piece of glass tubing of 4 mm. diameter tightly plugged with wool; after 50 days it was more acid than pH 6.6 and 5 c.c. required 0.2 c.c. N/10 NaOH to adjust its reaction to pH 7.2.

The growth obtained on good media when 5 per cent. of the fluid which

had become alkaline was added, was too sticky to remove from the surface of the agar; neutralisation of the fluid (to pH 7.2) before using it gave smooth creamy growth once more.

It appears, therefore, that exposure to the air quite rapidly causes a change in ascites fluid, which renders it more than ordinarily difficult to use and for that reason we abandoned it.

*Horse serum and laked horse blood.* The serum was obtained in the ordinary way by allowing sterile blood to clot. The laked blood was obtained by drawing horse blood into an equal volume of sterile distilled water, it haemolysed immediately, subsequently a clot separated and the haemoglobin stained serum was used to supply the accessory growth factors. Both these substances were added to the medium immediately before use in quantities varying between 1 and 5 per cent. and they stimulated growth nearly, but not quite, as well as human ascites fluid.

We discontinued their use because they exhibited a similar change to that described in ascites fluid and with the same effect, without presenting any other property of particular advantage.

*Formol serum* was prepared as described by Nicolle, Debains and Jouan (1918). Legroux (1920), using horse serum. It was added to our media immediately before use in the quantity directed and our results were less satisfactory than those obtained with ordinary serum or laked blood. This substance also became alkaline when exposed to air and again after neutralising.

It is a curious fact that formol serum prepared as directed does not clot when autoclaved, it becomes more opalescent and a smell of  $H_2S$  is evolved; and that whole serum to which 2 per cent. of formalin has been added does not coagulate when heated at  $100^\circ C.$ , or even when boiled, but when added to medium containing 2 per cent. of agar and our usual salts coagulation takes place on steaming or autoclaving.

*Extracts of red corpuscles*, prepared in the manner described by Agulhon and Legroux (1918), Legroux and Mesnard (1920), when added to media immediately before use gave results slightly inferior to those yielded by our ordinary media, to which the accessory growth factors were added before sterilisation. The only remark we have to add is that the addition of the extract before autoclaving only slightly reduced its efficiency. These extracts were designed to replace fresh blood in media on which it was required to grow Pfeiffer's bacillus.

*Gordon's extract of pea-flour* was used in preparing "Trypagar" as described by Gordon, Hine and Flack (1916). We did attempt a few experiments in which this extract was added to our media, but with little success as the results were always inferior to our standard method.

When this substance was used by Gordon and others, when the medium was supplied on a large scale during the epidemic of cerebro-spinal fever in the British Army, it was always accompanied by horse serum or laked rabbit blood, to be added to the medium before use. This fact made us less interested in it



than we would have been had it unfailingly grown the meningococcus in primary culture without the additional substances.

*Freshly drawn horse blood.* The addition of fresh blood to media is a well-established method of promoting the growth of delicate organisms and it is usual either to add it to the melted and cooled medium, or to smear the surface with blood, but each method suffers from inconveniences we wish to avoid. The coagulation of blood in the medium by heat has had its advocates. While certain of them are content with an opaque medium rather resembling a slab of chocolate, others have removed the coagulated protein by specialised methods with the idea of retaining the accessory growth factors, which otherwise, they claim, are destroyed or removed (Lloyd, 1916). The method we advocate, which has given results only surpassed by *freshly drawn* ascites fluid and perhaps the method we shall presently describe, depends upon the coagulation of fresh blood in the presence of agar and the subsequent removal of the clot, without interfering with the accessory growth factors required by the meningococcus in any degree we can detect. At the same time the method has this advantage, that no elaborate nor troublesome technique is required either for the removal of the coagulum or the sterilisation of the finished medium. When all the ingredients of the medium have been added and the melted agar has been cooled to 50°—55° C., 7 per cent. of freshly drawn horse blood is added, with stirring to make a homogeneous mixture, which is then gradually raised to 100° C. in the steamer. By keeping it at this temperature for 30 minutes a considerable shrinkage of the coagulum is produced. The agar is then allowed to cool and set and so is *left overnight*. We consider it to be important to let the agar set in the presence of the coagulated blood, as we have tried on many occasions the effect of removing the coagulum immediately after heating at 100° C. and without preliminary setting of the agar, but the result has always been most unsatisfactory. It is possible that the alteration of the physical state of the agar during setting plays some part in the process. The next morning the agar is melted, decanted off from the coagulum which will have contracted further and strained through surgical lint in a hot-water funnel; finally the clot is tipped into the filter and after draining the remaining medium is gently wrung out by twisting up the lint. After the reaction of the filtrate has been adjusted to pH 7.2, it is distributed as required and sterilised in the autoclave at 120° C. for 20 minutes. The resulting medium is glass clear, although there may be a small flocculent deposit which rapidly settles out, and the virulent meningococcus grows readily in primary culture. The removal of the coagulum is facilitated by carrying out the process in straight-sided enamelled pails, within which are placed closely fitting muslin bags, held in position by strips of cane. When coagulation has taken place the strips of cane are removed and the bag is gently drawn to one side; the bag holds back the clot while the medium is decanted and eventually, after draining, the bag and contained clot are wrung out into the lint filter.

We wish to comment on a few points arising out of this method:

The quantity of blood used is the maximum which we found gave us a manageable coagulum; greater quantities were inconvenient and did not seem to possess any advantage. Better results seem to have been obtained when the reaction of the medium in which the blood was coagulated had been adjusted to pH 7.0—7.2, rather than when it was definitely acid or alkaline.

We tried various methods of separating the coagulum, such as filtration through glass wool, paper and lint, also simple decantation, but we were unable to detect any difference in the growing power of the product. Filtration through lint possesses the great advantage of being a rapid process with very little loss of material.

Sterilisation by steaming at 100° C. on three successive days showed no advantage over autoclaving. In fact we find that this medium stands several autoclavings on different occasions at 120° C. for 20 minutes, without appearing to change in any way other than becoming clearer.

In the course of our work it has frequently been necessary to make a series of media, in small amounts, in which some one constituent varied. This was greatly simplified by the fact that the coagulation of blood in a solution of agar in extract, with the subsequent addition of the other constituents, was quite as effective as coagulating the blood in the otherwise finished medium.

We call this form of medium "*EB agar*" (Extract, Blood) and "*EDB agar*" when the digest has been added.

*Fresh heart muscle.* Under certain conditions it is difficult to get a sufficient quantity of freshly drawn blood to make large amounts of medium in the manner described and it occurred to us to try to use the heart muscle from which we make our extract and digest. But before proceeding we wish to repeat that we only use freshly killed meat.

Attention has already been drawn to the copious coagulum we observed in making our ordinary extract, when it was raised to 100° C., but we wish now to emphasise another closely related point. When the meat has been extracted at 70° to 75° C. for three hours the fluid is a rich tawny red colour, which changes when heated at 100° C. to a pinkish yellow, with the separation of the coagulum and on autoclaving a further change of colour to a bright yellow takes place, with the separation of a fine whitish precipitate. At each stage the fluid has a bright, crystal clear appearance.

We therefore filter the extract immediately after its three hours at 70° to 75° C., in the manner described under the method of making the extract, and into this filtrate we stir an equal volume of a 4 per cent. solution of agar in ordinary extract cooled to 70° C. The whole is then raised to 100° C. and treated strictly in the manner described for coagulating blood in the medium, with the exception that the muslin bags are dispensed with because there is not so much coagulum to remove. The resulting filtrate is not so clear as when blood has been used but its transparency can be much improved by entangling the fine coagulum in a precipitate of phosphates, as described under methods of

clearing medium. But we find it advisable not to clear the medium thoroughly as definitely better results are obtained when it is slightly cloudy.

This preparation, which we call "*EH* agar" forms the basis of our medium and it keeps well stored in this form after autoclaving. To it we add the desired quantities of digests and salts and adjust the reaction, to make the finished medium which we speak of as "*EHD* agar" (Extract, Heart, Digest). This medium is no whit inferior to "*EDB* agar," in fact we are rather inclined to judge it as better. The accessory growth factors in this form are resistant to autoclaving and we strongly recommend the method for making media for general purposes.

It is best to avoid autoclaving "*EH* agar" more frequently than is absolutely necessary as the finely suspended matter tends to agglomerate and settle out as a reddish deposit, leaving the agar perfectly clear and the growth obtained on this very transparent medium is erratic and in the case of highly virulent strains it may be uncertain. In this respect "*EHD* agar" differs from "*EDB* agar" which is unaffected by thorough filtration or the clearing effect of repeated autoclaving.

If, however, the deposit is added to part of a plate of perfectly clear "*EHD* agar," satisfactory growth occurs on that part of the surface overlying the deposit; and if the fine precipitate is evenly distributed through the medium before use, perfectly satisfactory growth is obtained over the whole surface. For this reason we are content at present with the slightly cloudy medium obtained by filtration through lint and do not clear it by means of the phosphate precipitate or any other method.

(i) *The influence of agar-agar.*

Certain influences of agar have been discussed (Section II) but there are a few other points worth considering. Agar in "powder" or other "specially prepared" form is less satisfactory than plain "bleached fibre" (China grass); principally because the prepared agar is much less constant in the character of the jelly furnished by different batches.

Although treatment with acid probably increases the imbibition of the agar, we have not observed any particular advantage exhibited by agar so treated.

There is, however, one disadvantage in washed agar used wet, that it absorbs and holds about eight times its dry weight of water in spite of squeezing it as hard as it will allow; when the concentration of dry agar used in the medium is 2 per cent., this water dilutes the extract used by 16 per cent., and in order to avoid this in experimental media we used to dry the agar after treating it with acid (0.01 per cent.  $\text{H}_2\text{SO}_4$ ) and washing until acid free, on the lines indicated by Cunningham (1919). We have, however, abandoned this process in favour of four washings with distilled water, wringing the agar as dry as possible in a clean cloth between each.

Up to the present we have not investigated the influence of agar on the growth of the meningococcus, although we have noted some observations

which tend to show that it is not the inert substance it is usually supposed to be.

The concentration of agar we favour in our medium at present is 2 per cent. of dry fibre, principally because this gives a sufficiently firm jelly, enabling us to scrape the growth off its surface without risk of taking up pieces of agar which would falsify our weighings. Nevertheless a more ready growth is obtained on lower concentrations and the work of Jenkins (1921) on the gonococcus is instructive; although we are not inclined to confine the activity of agar to concern moisture only.

In our opinion the influence of agar is one of the difficult factors to control in making media and concerning which very little is known.

(j) *Summary of the method of making "EHD agar."*

(1) *The extract.* Ox heart muscle, which has been killed not more than 24 hours, is freed of fat, minced, but not too finely and suspended in twice its weight of distilled water in an open can in a steamer and its temperature raised gradually to 70°–75° C., with occasional stirring to prevent the surface layer getting too hot. This temperature is controlled by a thermometer in the mixture. After 3 hours the temperature is allowed to rise to 100° C. for 15 minutes to get a firm coagulum. It is then filtered through its own meat, as described under (a) of this section. *Do not filter through lint or paper.* This furnishes the extract, which can either be autoclaved and stored, or used immediately to make a 4 per cent. solution of agar.

(2) *The "EH agar."* Proceed as in making the extract until the end of the three hours' heating at 70° to 75° C., then filter through the meat. To this filtrate is added an equal volume of the 4 per cent. solution of agar in extract after cooling it to 70° C.; this mixture is returned to the steamer and raised to 100° C. for one hour. It is then *left to set* and stand overnight. The next morning it is melted and strained through lint. This filtrate is the "EH agar," which can be autoclaved and stored if not required immediately.

(3) *The finished "EHD agar."* To the required amount of melted "EH agar" are added: 0.25 per cent. NaCl, 0.02 per cent. KCl, 0.01 per cent. CaCl<sub>2</sub> and the desired concentration of digest (Section V). The reaction is adjusted to pH 7.2 and the medium distributed as required and autoclaved at 120° C. for 20 minutes.

(4) *The digest.* The residual meat and fine coagulum filtered off from the extracts are suspended in a quantity of  $N/100$  HCl equal to the weight of original raw meat. This bulk is raised to 100° C. and then autoclaved at 130° C. (25 lbs. to the sq. in.) for 30 minutes, then cooled to 37° C., incubated 24 hours and its sterility assured. Then 2 per cent. of sterile pancreas extract is added and left in contact with the HCl for 5 to 15 hours to activate the trypsin, after which 0.8 per cent. anhydrous Na<sub>2</sub>CO<sub>3</sub> is added in the form of a sterile 32 per cent. solution. This strength of Na<sub>2</sub>CO<sub>3</sub> readily dissolves at 37° C. Digestion is allowed to proceed until a "Sørensen figure" of not less than 20 is produced

(see (b) of this section). When this result has been obtained, 2 per cent. of 10N HCl is added and the digest autoclaved and while still hot filtered through paper in a hot funnel. The acidified digest filters quite as rapidly as it is slow when alkaline. At least one advantage of strict sterile precautions is that the digest retains a pleasant meat-like smell throughout the process, both in the alkaline and the acid condition. Any objectionable smell or traces of  $H_2S$  detected shows that contamination has occurred. The filtrate is put into clip-top milk bottles and autoclaved at  $120^\circ C.$  for 20 minutes, and repeated autoclaving has no apparent deleterious effect.

#### V. THE INFLUENCE OF THE CONSTITUTION OF THE MEDIUM ON VIRULENCE.

Attention has already been drawn to our observation that the killing power of a given strain of meningococcus was greater or less according to the medium on which it was grown (Section IV (b)) and that we were then inclined to attribute the alteration in the physiological state of the parasite, resulting in a lowered virulence, to the contamination of our digest by certain other organisms during digestion. We have not been able to demonstrate that such contamination has this definite effect.

Nevertheless this primary observation induced us to investigate the relation of growth to medium, not so much from the point of view of the mass yielded by the medium as the physiological state of the growth, measured by its capacity to kill mice inoculated intraperitoneally.

This line of research has yielded what we venture to think are important results and we propose to consider them in some detail.

The killing power of a culture is estimated by determining the Minimal Lethal Dose in the way described by Murray (1924, p. 177), and, in our attempt to determine what constitutes a good medium, although we regard variation in killing power as more important, we have not neglected to observe the mass of growth yielded. At the outset we discontinued the use of contaminated digests and confined our attention to those made with strict sterile precautions, with the intention of reverting to our first observation by means of controlled growth, of known organisms, in digests which proved to give good results.

As has been stated, our early sterile digests had a much lower "Sørensen figure" than did the contaminated ones and our first endeavour was to produce sterile digests with a concentration of amino-acids quite as high as that which the contaminated digests appeared to possess. This was particularly desirable as we thought that the general amino-acid concentration, indicated by the "Sørensen figure," might afford a means of standardising media both from the point of view of virulence and growth.

The following experiment bears directly upon these ideas and the results are very instructive. We prepared three solutions:

(a) In 3000 c.c. of extract we dissolved 2 per cent. of washed and dried agar, 0.5 per cent. NaCl, 0.125 per cent.  $CaCl_2$  and adjusted the reaction to

pH 7.2. This solution was then filtered through lint which had been washed with boiling distilled water.

(b) 250 c.c. of digest No. 109, with a Sørensen figure of 32.0 c.c. N/10 NaOH on 10 c.c. of digest, was treated like the extract in (a).

(c) 650 c.c. of digest No. 118, with a Sørensen figure of 13.1 c.c. N/10 NaOH on 10 c.c. of digest, was also treated like the extract in (a).

These three solutions were then mixed in the proportions shown in Table VI to make media *A*, *B* and *C*. The reaction was checked with the interesting result that it needed readjusting in each of the *B* and *C* media. These were then distributed in 100 c.c. volumes in marked flasks and autoclaved at 120° C. for 20 minutes. When the media were used they were melted and cooled to 50° C. and 5 per cent. of ascites fluid was added, three plates of each were poured, all of which were inoculated consecutively from a growth of a fairly virulent strain, first generation from an egg culture on a medium known to give good results. A sixteen hours' growth on the various media was inoculated intraperitoneally into mice (= 2nd generation from the egg culture = 1st generation on the special media), two mice were used for each dose. The result of this experiment is shown in Table VI.

In considering these results it must be observed: Firstly, that the accessory growth factors were supplied by ascites fluid and for that reason growth was obtained on medium *A*; also that the ascites fluid used was then beginning to influence the sticky character of the growth (Section IV (*h*)). Secondly, that the concentration of NaCl added may be considered to be high (Section IV (*d*)), and that we did not take into account the salt content of the digests. Bearing these points in mind, the physical character of the growth is capable of interpretation. With regard to the mass of growth yielded by the various media, it is evident that the addition of digest not only increases the yield compared with medium *A*, but that there is some suggestion of an optimal range of concentration, which is more marked in the *C* media than the *B*. Further, the difference between the *B* and *C* groups of media bears no marked relation to the "Sørensen figure" increment due to the digest. At the same time there appears to be a definite decrease in the percentage of moisture in the growth yielded by the high concentrations of digest compared with the low, but this may be due to the contained salts (see Table V).

The outstanding feature of the experiment is the behaviour of the cultures towards mice. This shows definitely that there is an optimal range of concentration of digest, which alone affords the meningococcus such conditions that allow of its development of something essential to its manifestation of a parasitic existence. Furthermore, this "virulence range" appears to be independent of the increment of the "Sørensen figure" due to the digest, and, except in the case of the *C* media, it does not show any marked relationship to the mass of growth yielded. On the grounds of deficiency, it is not surprising that the effective range of the digest should exhibit a low limit, but it could not have been expected that an upper limit would be found; this suggests the

presence of an inhibition factor overpowering the effect of the presence of a sufficiency of the necessary substances. Yet this upper limit is definitely marked in both the *B* and *C* media.

Table VI.

Medium No.	% concentration in (a) of		Increment of Sørensen figure in c.c. $N/10$ NaOH on 10 c.c. due to added digest	Generation	Growth in mgms. per sq. cm. of 5 successive generations on each medium		Dry growth as a % of moist	Character of growth	Killing power of the first generation on each medium (2 mice to each dose)	
	(b)	(c)			Moist	Dry			Dose in mgms. of living culture per 20 gms. mouse	Result
A	{	0	0	1	1.02	0.18	17.5	All slightly sticky	8	Both lived
				2	0.80	0.13	16.7		{	"
				3	0.54	0.09	16.7			
				4	0.60	0.10	17.2			
				5	0.95	0.17	18.2			
B 1	{	2	0	1	1.35	0.25	18.2	All slightly sticky	8	Both died
				2	1.23	0.20	16.2		{	"
				3	1.23	0.21	17.3			
				4	1.16	0.20	17.7			
				5	1.28	0.26	20.0			
B 2	{	8	0	1	1.32	0.25	18.7	Sticky	8	Both died
				2	1.61	0.31	18.9		{	"
				3	1.50	0.28	18.6			
				4	1.53	0.28	18.8			
				5	1.46	0.30	20.5			
B 3	{	32	0	1	1.43	0.29	20.3	Very sticky (2nd gen. too sticky to scrape properly)	8	Both died
				2	(0.9)	(0.15)	16.9		{	One died
				3	1.37	0.25	18.2			
				4	1.00	0.20	20.2			
				5	1.55	0.31	20.0			
C 1	{	0	5	1	0.99	0.20	20.1	Sticky	8	Both died
				2	0.81	0.13	16.4		{	Both lived
				3	1.36	0.26	19.2			
				4	1.28	0.24	18.6			
				5	1.71	0.32	18.4			
C 2	{	0	20	1	1.58	0.31	19.8	Very sticky (5th gen. too sticky to scrape properly)	8	Both died
				2	1.55	0.33	21.1		{	"
				3	1.13	0.23	19.9			
				4	1.35	0.30	22.2			
				5	(0.8)	(0.15)	19.2			
C 3	{	0	80	1	—	—	—	Sticky	8	One died
				2	1.10	0.25	22.7		{	Both lived
				3	0.74	0.19	25.5			
				4	0.87	0.22	25.0			
				5	0.70	0.19	26.6			

In each of eight experiments performed, using different digests, we obtained results strictly like those detailed above. Experiments of this nature present many difficulties when working with the meningococcus, besides involving the use of a considerable number of mice and it is not always possible to insure that all the factors contributing to success will be in working order at the desired moment. In our experience the "virulence range" of a digest is difficult to determine if the strain used for the test is of a very high order of virulence, as the range may then appear to be unnaturally extended. In practice we

prefer to employ and get best results with a strain 2 mgms. of which kills 20 gms. of mouse within 14 to 48 hours, when grown on the medium of optimal concentration of digest. A strain exhibiting a Minimal Lethal Dose of 4 mgms. is often useful, but one with a Minimal Lethal Dose of 1 mgm. kills when grown on media which are not the best. We believe that the medium giving best results with an organism of failing virulence, probably is also the best for the very virulent.

The highest "Sørensen figure" we have met with in our sterile digests was one of 49; the lowest observed effective concentration of this digest, as determined by virulence, was 1.8 per cent., a concentration of 0.54 per cent. gave a good yield of growth but of markedly reduced virulence; the highest effective concentration observed was 15 per cent., but higher concentrations were not tried for virulence, but 21 per cent. gave a reduced yield of growth.

The digests exhibiting a "Sørensen figure" of not less than 20 had a wider effective "virulence range," particularly noticeable in that it extended to much lower concentrations of digest in the medium, than those other digests whose "Sørensen figure" was in the region of 12 to 15. It is for this reason that we consider digestion sufficiently advanced for our purpose, only when the "Sørensen figure" has a value of not less than 20 (see Section IV (c)).

One other experiment may be quoted in detail, as it illustrates our general experience of the relation of growth to virulence in the type of experiments now under consideration. In this case the basis of the medium was "*EB* agar" (Section IV (h)) and the salt concentrations were 0.25 per cent. NaCl, 0.01 per cent. CaCl<sub>2</sub> and 0.02 per cent. KCl (Section IV (d)). The concentrations of digest and general results are shown in Table VII.

Table VII.

Medium	% concentration of digest 150	Increment to Sørensen figure due to the digest in c.c. <i>N</i> /10 NaOH to 10 c.c. of medium	Arithmetical mean of			M.L.D. in mgms. per 20 gms. of mouse (1st gen. on medium)	
			Moist growth in mgms. per sq. cm.	Dried growth in mgms. per sq. cm.	Dried growth as a % of moist growth		
<i>A</i>	1	0.24	2.8	0.49	18.6	8	
<i>B</i>	2	0.48	3.2	0.57	17.8	2	
<i>C</i>	4	0.96	3.2	0.58	18.5	2	
<i>D</i>	8	1.92	3.0	0.55	18.1	4 ( $\pm 2$ )	
<i>E</i>	16	3.84	2 plates were contaminated, 1 used for mice. Further generations not continued				8

It is evident, that although a marked difference in killing power exists between the growths obtained on the various media, the differences in the yield of growth per unit area of medium are relatively slight and certainly would not be appreciated by inspection. For reasons such as the result of this experiment, we do not believe that the yield of growth is necessarily a reliable index of a good medium for highly specialised parasitic bacteria. The variation in the percentage of moisture, which is the chief source of error in carefully measured doses of weighed growth, cannot be held accountable for the differences in killing power of the cultures.



As the result of this enquiry, we were naturally forced to the conclusion that the standardisation of a medium for the meningococcus, is a much more difficult and delicate matter than our previous experience and the literature of the subject had led us to believe.

By taking into consideration the "virulence range" of the digest and by adjusting the various other factors discussed in Section IV, we evolved our "*EDB/V*" and "*EHD/V*" medium (*V* = virulence). The yield of growth and degree of constancy of this medium has been discussed in Section III (Table I), where it is shown to be the best medium we know at present.

Although we have shown quite definitely that this "virulence range" exists, we have no idea of its underlying cause; and we have not succeeded in devising a sufficiently delicate test whereby to determine the optimal point in the range. Our present practice, in making the medium, is to use an amount of digest a little higher than the lowest effective concentration determined by the killing power of the cultures on mice. This requires that each digest has to be titrated by experiments using the Minimal Lethal Dose of a suitable culture as the indicator, and, although it may be considered a troublesome necessity, we are convinced that it is well worth while. In order to minimise the labour we make amounts of digest varying between two and six litres, and, since the required concentration is only in the region of 2 per cent. of digest, these large volumes make a considerable quantity of effective medium.

We may now reconsider the question raised concerning the effect of contamination of the digest in the course of its manufacture. We took a sterile digest, the "virulence range" of which we had determined, neutralised and contaminated a portion of it with *B. subtilis*. That organism would only grow with great difficulty in the concentrated digest, although abundant, typical growth was obtained in dilutions. In time, however, sufficient growth had taken place for a deposit of fluffy balls of bacilli to accumulate at the bottom of the vessel, but no surface film was formed, though abundant typical growth resulted with subculture on to ordinary media. This purposely contaminated digest was then filtered through a sterile Pasteur-Chamberland candle "*F*" and the filtrate was used in varying concentrations to make media. So far as we could discover, the "virulence range" had not been altered by the contamination. The experiment was repeated with the same result. But two things must not be lost sight of: firstly, the degree of contamination was comparatively slight for a freely growing organism like *B. subtilis*; and, secondly, our original digests were contaminated by a varying mixture of organisms, including anaerobes.

Before proceeding further with this type of experiment we thought fit to examine some of the old "non-sterile" digests we had kept. Varying amounts of seven different digests, which had been used for making "*EDB/N*" medium, were pooled; in a portion of this we dissolved 2 per cent. of dry agar and this solution was mixed with *EH* agar in the proportions shown in Table VIII. In adding the salts allowance was made for the NaCl content of the digest and

the final concentrations were 0.25 per cent. NaCl, 0.01 per cent. CaCl<sub>2</sub> and 0.02 per cent. KCl. The media were inoculated with a strain of good killing power, from a culture first generation from egg (M.L.D. = 2.0 mgm. of growth on *EHD/V* for 20 gms. of mouse).

Table VIII.

Medium	% concentration of pooled "non-sterile" digest	Increment of Sørensen figure due to digest	Growth in mgms. per sq. cm.		Dried growth as a % of moist	M.L.D. in mgms. per 20 gms. of mouse (= 1st gen. on these media = 2nd gen. from stock egg)
			Moist	Dried		
A	0.5	0.18	2.2	0.32	14.8	4
B	2	0.71	2.5	0.38	15.4	2
C	8	2.85	3.0	0.49	16.6	2
D	32	11.39	2.1	0.40	19.2	4

From the result of this experiment (shown in Table VIII) it is quite evident that the pooled digest has a "virulence range," in spite of having been contaminated during digestion. But we now regret that we did not examine each of the digests separately, for it is probable that they had individual differences.

Our original conception that contamination altered the digest, to an extent which interfered with the virulence of the cultures grown on it, is shown by the above experiment to be without foundation.

The difference we originally observed between certain "contaminated" and "sterile" digests, in regard to the virulence of the growths they respectively yielded, is readily explained by the fact that the media were then made by adding the amount of digest required to give a definite increment in the Sørensen figure. This method we have since shown to be unreliable as the Sørensen figure bears no relation to the "virulence range" of the digest; this is amply demonstrated in Tables VI, VII and VIII. Even though contamination of the material during the course of digestion does not decrease its value, we strongly advise the adoption of strict sterile precautions, if only to avoid the very objectionable smell which often results from contamination. Sterile digests have, in fact, a very pleasant and appetising smell.

#### VI. THE RELATION OF MEDIUM TO VIABILITY AND MAINTENANCE OF VIRULENCE.

In the foregoing sections we have discussed the influence of the constitution of the medium upon physiological characters of the meningococcus which can be measured in terms of reproduction *in vitro* and adaptation to a parasitic existence. In the present section we wish to consider briefly a few scattered observations on the duration of the life and the virulence of cultures of that organism *in vitro*.

It is generally agreed that the life of a culture of meningococcus on the ordinarily used media is short and it is very commonly stated that the culture may die within 48 hours. Various media have been considered satisfactory because stock cultures remained alive for one or two months, and it is quite

likely that the strains used had been subjected to subculture before the test was performed. In our experience, before we had investigated the facts detailed in the preceding sections, Dorsett's egg medium maintained the life of meningococcal cultures better than the other media we had tried. A large proportion of cultures on egg could be recovered when they were a year old and only an occasional one died in less than six months, but absolute certainty only prevailed with monthly subculture. In any case direct subculture from an egg slope a month or more old on to agar media was often a matter of difficulty, it frequently failed to give any growth and quite commonly only a few scattered colonies resulted, even with quite heavy inoculation. Subculture from egg to egg gave better results, but, even so, the appearance of only a few scattered colonies was a far too frequent occurrence; and these scattered colonies commonly had to be subcultured daily for a few generations before vigorous growth obtained. Thus it became our usual practice, when growth of a particular strain living on egg was required on agar medium, to resort to an intermediate young subculture on egg.

When we first observed the difference in virulence of cultures grown on particular samples of *EDB/N* and *EDB/S* media, we were examining the influence of repeated subculture on the growth of the meningococcus. Two series of subcultures were being run at the same time: one at 12 and the other at 24-hourly intervals. In the 24-hourly series there was a marked initial lag with each subculture, but in the 12-hourly series growth appeared more and more rapidly with successive subculture, until, after a few generations, 4 hours' incubation yielded a considerable growth. In the course of 36 days the *EDB/N* medium with which we had started came to an end and we proceeded with a batch of *EDB/S*; these were the same batches of media on which we had noticed the difference in virulence, but we did not at first observe any marked difference in the growth as these generations were carried out on slopes, in test-tubes plugged with wool in the ordinary way. We had previously noticed that cultures which were kept at 37° C. for 14 to 30 days, occasionally gave rise to scattered colonies superimposed on the old growth, so these various cultures were all kept for a month to watch for this secondary growth, without any precautions to prevent drying of the medium. Out of 108 cultures grown on *EDB/N* (No. 86) (71 from the 12-hour and 37 from the 24-hour series) only 4 showed secondary growth; whereas of 37 cultures grown on *EDB/S* (No. 88) (25 of the 12-hour and 12 of the 24-hour series) every one gave good secondary growth.

In view of the experiments described in Section V, the outstanding feature of this observation depends upon the fact that growth yielded by *EDB/S*, No. 88, was virulent, whereas the same strain grown on *EDB/N*, No. 86, failed to kill mice. Backed by our observation on virulence in relation to these media, secondary growth immediately became a character of importance in our eyes, and caused us to think of the possibility of making a medium which would maintain a culture alive for a considerable time without loss of virulence.

At this time we thought these important differences might be due to the digest having been contaminated or not during its preparation and we resolved to test the viability of cultures on *EDB/N* and *EDB/S* media, with the result that in this case the non-sterile proved to be the better, but still fell far short of egg for this purpose, as is shown in Table IX.

Table IX.

Medium kept at 37° C. with waxed plugs	Subcultured from	Number of cultures	Number shown to be alive 9 months later, by subculture on <i>EHD/V</i> medium
<i>EDB/N</i> No. 72	Bacteriolysis Expt. 50, XVII	24	14
<i>EDB/S</i> No. 138	Bacteriolysis Expt. 50, XVII	26	7
Egg	<i>EDB/N</i> 72	18	18
Egg	<i>EDB/S</i> 138	16	15

Media 72 and 138 showed an identical increment in the "Sørensen figure" due to added digest; but there is considerable evidence that the concentration of digest 122 used for making No. 138 and other media was not favourable for virulence, and some evidence that No. 72 was a better medium from this point of view. No very precise information on this point is available because we had not yet recognised the principle of the "virulence range."

The recognition of the "virulence range" was immediately applied in the form of our early *EDB/V* medium and when this was used we observed that there was a rapid regrowth of the meningococcus over the area from which the original growth had been cleanly removed. This observation was followed by the experiments relating to inorganic salts in the medium (Section IV (*d*)) and the relation of regrowth of the culture to potassium salts was consequently noticed.

Previously we had tried a medium described by Wadsworth (1903), a weak agar jelly containing about 75 per cent. of serum or ascites fluid, in which the pneumococcus maintained its virulence at a constant level for several weeks (Wadsworth and Kirkbridge, 1918); some cultures of meningococcus died quite soon and others survived for a long time in this medium. But we were struck by the advantages of the weak agar jelly and the benefit of being able to dispense with waxed plugs. We therefore made *EDB/V* medium, containing the required amount of digest and salts but only 0.5 per cent. of agar; this medium we call "*F*." In order to have present the accessory growth factors required by the meningococcus, our practice is to dilute "*EB*" or "*EH* agar" with extract to which we have added the required amount of digest and salts, and, after adjusting the reaction, to distribute it in wool-plugged tubes under a layer of liquid paraffin and autoclave it.

We have not had this medium in use sufficiently long to be able to discuss its properties fully, but we may say that it promises well, for the following reasons: The meningococcus grows readily in it in primary culture from cerebro-spinal fluid and a profuse growth is obtained on subculture on to our ordinary medium (*EHD/V*), even when the culture is five months old. The

Minimal Lethal Dose of this culture was 2 mgm. for 20 gms. of mouse when put into "F" medium and it showed no alteration in killing power in four months; after five months it killed more often than not in a 2 mgm. dose (slightly irregular) and with absolute certainty in a 4 mgm. dose for 20 gms. of mouse. This is a considerable improvement on our experience with egg medium. Furthermore we have never worked with such virulent strains as those isolated on "F" medium.

During the last three years we received seventeen cultures of freshly isolated strains of meningococcus from private friends and as the result of an appeal by the Secretary of the Medical Research Council and the Principal Medical Officer of the Ministry of Health. These were grown on various media of which we know nothing, nor do we know how often they had been subcultured before we received them, but the relation of the Minimal Lethal Dose of the cultures to the medium on which they were sent to us is set out in Table X and has certain points of interest.

Table X.

Minimal Lethal Dose for 20 gms. of mouse	>8 mgms.	8 mgms.	4 mgms.	2 mgms.	Totals on each medium
Dorsett's egg	2	2	0	3	7
Inspissated serum	6	1	0	0	7
Trypagar	0	0	0	1	1
Unknown agar	0	0	0	1	1
EDB/N	0	0	1	0	1
Totals of each M.L.D.	8	3	1	5	17

The striking feature of these results is that no strain received on inspissated serum was virulent; but the other figures are difficult to interpret without further investigation of the problem.

We are greatly indebted to Professor H. R. Dean for his personal appeal to a wide circle of workers, asking them to collect primary cultures on our media and they have kindly consented to help us in this direction. During the last three months we have received ten suitable cultures on "F," egg and serum; each set having been inoculated direct from the same sample of cerebro-spinal fluid. Up to the present all the cultures on "F," nearly all on egg, and certain of the serum cultures have been virulent. Of these two were received from the same source as the serum cultures given in Table X.

It is yet too early to discuss these results; but at present it appears that the *primary culture* may be virulent even when the medium is not the most suitable, although on subculture its virulence may be lost. Of the virulent cultures shown in Table X, we know that the one we received on "Trypagar" was inoculated direct from the cerebro-spinal fluid.

There is one other aspect of the maintenance of virulence *in vitro* which deserves consideration. It is well known that many parasitic bacteria lose their virulence if frequently subcultured at intervals of 24 hours and that the meningococcus is particularly apt to behave in this way. It is especially interesting, therefore, to notice the relation of killing power to successive

subculture at 24-hourly intervals on *EHD/V* medium and the results of two such experiments are given in Table XI.

Table XI.

Experiment	Generations at 24-hourly intervals	Dose of living meningococci per 20 gms. of mouse (2 mice were inoculated with each dose)				
		8 mgms.	4 mgms.	2 mgms.	1 mgm.	0.5 mgm.
A	1	+	+	+	+	
	2		+	+	+	
	4		+	+	+	
	5	+	+	(+)(+)	(+)(+)	(+) 0
B	1		+	+	+	
	2		+	+	+	
	3	+	+	+	+	+
	4	+	+	(+) 0	(+)(+)	
	5	+	+	+	+	
	6	+	+	+	+	

+ = one mouse died in under 48 hours.

(+) = one mouse died between 48 and 86 hours.

0 = one mouse definitely survived.

Blank space = dose not tried.

These experiments suggest that it may yet be possible to produce a medium and a method of using it, which will allow of repeated subculture without loss of virulence. The fluctuations in the time the mice took to die gains in interest when compared with the fluctuations in yield of growth shown in Table II (Section III), for here again the rise and fall may be irregular after the first two generations. It is conceivable that this fluctuation in killing power and yield of growth, may be determined by the proportion of dead cocci contained in a given mass of growth; this interpretation is particularly suggested by the fact that the mass representing one Minimal Lethal Dose of killed cocci, when the organisms are entire, is very many times greater than that representing one Minimal Lethal Dose of the given living meningococcus culture.

We readily admit that the observations contained in this section are most incomplete, but, at least, they indicate that much may yet be done by a thorough investigation of the influence of medium on the maintenance of viability and virulence of cultures *in vitro*.

## VII. DISCUSSION.

Dopter (1921, p. 416) remarks, that the immunisation of animals against the meningococcus is a very delicate process which presents many technical difficulties and this observation is emphasised by his discussion of the methods advocated by eminent authorities. There appears to be no doubt that the immunisation of horses, with the object of producing a potent therapeutic anti-meningococcal serum, is by no means accomplished with any degree of certainty. Flexner, Dopfer, Gordon, Nicolle and others, have produced unassailable evidence that a highly potent therapeutic serum can be produced occasionally, but it is quite evident that failures have been a common experience. Similar failures appeared to us to call for a close study of the characters

of meningococcal antigens and the present paper deals with part of this investigation.

A study of the literature of anti-meningococcal serum, reveals no convincing evidence of any character which might serve as a guide in the production of a successful serum. The virulence of the cultures used as antigens does not appear to have been investigated and the explanation of this undoubtedly lies in the fact that it is generally admitted that the attempted titration of meningococcal virulence has resulted in failure.

In this paper we have discussed in detail some of the inherent difficulties presented by the cultivation of the meningococcus *in vitro*, and, although we cannot claim to have made an entirely satisfactory medium, it will be admitted that we have established that the constitution of the medium exercises an important influence on the "virulence" of the culture.

We have no evidence to show how important it may or may not be, to use only highly virulent meningococcal cultures as antigens for the production of potent therapeutic serums. Those concerned with the production of serums commonly express as their opinion, that it is desirable to use only freshly isolated strains, but judging by their behaviour towards mice, the freshly isolated strains shown in Table X of this paper are very different organisms from those we are obtaining in primary culture on our "*F*" medium. Thus, "freshly isolated strain" becomes a term of no exact meaning without qualifying it by describing the properties of the medium. Possibly "freshly isolated" strains are more likely to represent correctly the prevalent agglutinable types.

A few months ago we started to immunise horses in terms of the degree of virulence of cultures (titrated in mice) and it is a matter for regret that this part of our investigation had to be abandoned, through circumstances not under our control, just at a time when we appeared to have mastered some of the chief difficulties in the manipulation of meningococcal virulence.

The work of Cotoni, Truche and Raphael (1922), although dealing with the pneumococcus, bears on this question with considerable weight. In discussing the protective power of active immunisation with vaccines and the production of potent protective serums, they repeatedly emphasise that satisfactory results have been obtained only when very virulent cultures were used. They even go so far as to say (p. 82) "It is impossible to obtain an active serum with an avirulent or slightly virulent pneumococcus" and (p. 78) "To prepare a multivalent serum it appears to us to be an absolute necessity to use a very virulent pneumococcus."

There is no doubt that the position of anti-meningococcal therapeutic serum still is most unsatisfactory. The identification of agglutinating types and the application of this knowledge was undoubtedly a step forward on the evidence of Gordon, Nicolle, Netter, but the production of a potent serum of any type is not a certainty. The use of virulent cultures as antigens may prove to be merely "clutching at a straw," but whether this is the case or not can only be known when the method has been tried. In any case it is

important to remember that a fundamental principle of Pasteur's active immunisation was to use successive doses of increasing virulence, and that we can make no claim to greater success to-day than he achieved.

The evidence we have brought forward is a step towards making it possible to test whether the virulence of a meningococcal culture bears any relation to its antigenic capacity. But for the present we must content ourselves with agreeing with Nicolle and Césari (1924, p. 76) that this information is most desirable.

We suggest that the experiments described in this paper show that the general question of culture media needs further investigation. There are certainly components in media which influence to a profound degree the physiological state of the micro-organisms grown on them. Since writing this paper we have read the interesting work of Felton and Dougherty (1924) who show that the virulence of a strain of pneumococcus can be enormously increased by repeated subculture in milk at intervals of 2 to 8 hours (p. 141), and that although similar subculture in meat extract or ordinary broth results only in lowering the virulence, an increased amount of "Peptone" will allow of it being maintained (p. 164). This work raises the hope that with further experiment it may be possible to maintain, or even increase, meningococcal virulence by suitable cultural methods.

It still remains an open question, whether the cultures we are accustomed to use may be considered to be normal healthy organisms and representative of their kind. That is to say, we do not know whether the physiological state of the organisms in our cultures *in vitro* is identical with that of those actively causing disease in their natural host. Up to the present we have not been able to detect a difference between naturally virulent primary cultures and those in which the virulence has been raised by the method described by Murray (1924, p. 194), but in both cases we are dealing with cultures.

In this respect it is interesting to note that we have seen, on several occasions, a definite early purulent meningitis in mice that have been inoculated intraperitoneally, with cultures grown on our medium standardised by virulence tests. Microscopically the scanty pus was quite typical of the disease and the meningococcus was recovered in culture. One of our most striking instances of meningitis in a mouse resulted from a culture whose virulence had been raised by the *in vitro* method. But since we have not made a systematic investigation of the point, no definite conclusion can be drawn.

The special advisory committee upon bacteriological studies of Cerebro-spinal Fever during the epidemic of 1915 (Medical Research Committee, 1916, p. 20), in discussing culture media for the growth of the meningococcus, paraphrase Gordon (1916) as follows:

"The requirements of a good routine medium for the purpose have been stated as follows:

1. The meningococcus must grow on it readily and with certainty.



2. It must be easily and cheaply made and must not involve ingredients now difficult to procure in this country.

3. It must be of such a nature that it can be stored and sent out in bulk from a central laboratory.

4. It should preferably be transparent.

5. The viability of the meningococcus on it should be as prolonged as possible."

Our *EDB/V* or *EHD/V* medium fulfils all these requirements quite as well and certain of them better than any of the many media we have tried. Furthermore, the evidence we have brought forward in this paper allows us to add another very important requirement: That the medium must allow the meningococcus cultures grown on it to develop and maintain the physiological characters contributing to virulence. We might also add requirements relating to the physical characters of the growth: particularly percentage of adventitious moisture.

We are only too well aware of the tentative nature of many of our observations and that a fuller investigation of many points would add to the value of our paper; but work of this nature could easily be prolonged for an indefinite time and still remain incomplete. It will be admitted, perhaps, that we have at least recognised a problem requiring solution and taken steps in a direction from which useful results may be forthcoming.

In conclusion we wish to thank those who have kindly sent us cultures and all who have promised to do so should they get cases. We are particularly indebted to Professor H. R. Dean and Dr Duncan Forbes, who have kindly made personal appeals for cultures to be sent to us on our own media.

Finally we wish to thank J. Bain and E. Pleasance for their painstaking and willing assistance, which has contributed so largely to the success of the work.

#### VIII. CONCLUSIONS.

(1) That it is extremely difficult to make any two batches of a given medium sufficiently alike to obtain identical cultural results with the meningococcus.

(2) That this is largely due to our insufficient knowledge of

(a) the raw materials required, and

(b) the relative concentrations of the ingredients necessary, to afford the optimal conditions required by the organism to develop their natural physiological state essential to a successful parasitic existence.

(3) That the yield and physical characters of the growth are insufficient criteria whereby to judge a given medium as good or bad, since the killing power of a culture appears to be to a certain degree independent of these. That the present state of our knowledge requires that several characters be examined simultaneously in judging a medium for the meningococcus; such as:

(a) the alacrity with which growth takes place,

(b) the yield of growth,

- (c) the physical characters of the growth,
  - (d) the viability of the culture,
  - (e) the virulence, and
  - (f) the maintenance of virulence with age and subculture.
- (4) With the kind of medium considered in this paper, a good deal of truth is expressed by saying:

- (a) that the virulence of the culture is chiefly affected by substances contributed by the digest;
- (b) that the added inorganic salts and possibly the physical state of the agar, contribute largely to the physical characters of the growth; although other factors are also concerned;
- (c) that the yield and viability of the culture is determined by all the factors being correctly balanced.

(Elimination of by-products has not been discussed.)

(5) That there is an optimal range of concentration for tryptic digest of heart muscle, over which virulent cultures of the meningococcus are obtained.

(6) That media made with due consideration of the "virulence range" of the digest used, are favourable to the viability and maintenance of virulence of the culture.

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## THE BACTERIOLOGY OF HUMAN MILK.

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THIS enquiry into the bacteriology of human milk was prompted by our recent investigation of a case of entero-colitis in a breast-fed infant. In this case an abundant growth of a streptococcus was obtained from the faeces of the infant which was identical as far as we could determine with a streptococcus isolated from the mother's milk.

On turning to the literature, remarkably little work seems to have been done on this subject; in fact most of the modern text-books of paediatry either ignore it completely or dismiss it in a few lines. There is no reference to the subject in the English text-books to which we have referred. In America, Holt and Howland (1922, p. 146) write: "Under normal conditions woman's milk may contain a few bacteria; they are chiefly cocci derived from the internal milk ducts and are of no importance. In suppurative inflammation of the mammary gland, numerous bacteria may be found in the milk; also in some cases of puerperal sepsis. Tubercle bacilli have been demonstrated by Roger and Garnier in the milk of women with advanced tuberculosis, but ordinarily they are not present unless the gland is the seat of the disease."

Hess (1923, p. 282) believes that infection of the infant may be caused by lack of asepsis in the care of the maternal breasts.

Von Reuss (1914) states that it is impossible to prevent bacteria entering the oral cavity and intestine of the infant, even with the most extreme care on the part of the attendants, because they may come from the milk ducts or the genital canal of the mother. He also says that infections due to streptococci derived from the maternal circulation before birth (which in itself may be the cause of premature birth), or to secondary infections through the mother's milk, lochia, or other products entering the gastro-intestinal tract by way of the mouth, are among the most virulent. Von Reuss considers that if there is a possibility that the mother's milk is the source of infection, it should be obtained from another source, or sterilised.

Köstlin (1897) investigated the secretion of the breast in 100 pregnant women, and the breast milk in 137 lying-in cases. In the 100 pregnant women he found *Staphylococcus albus* present in all cases, *S. aureus* in 23, and "bacilli" in 25. In 14 cases the secretion was sterile. In the 137 cases from women during the puerperium *S. albus* was present in 132, *S. aureus* in 79 and bacilli in 22.

Cohn and Neumann (1891) collected breast milk after disinfection of the nipple with sublimate, alcohol, and ether, into sterile test-tubes; from 5 drops to 1 c.c. of milk were taken and the first and last portions were examined. They found that the milk from healthy breasts at which strong infants had just suckled contained very few or even no bacteria, but that in cases where the children were weakly or diseased the bacterial content varied from 16 to 150 colonies. If suckling was discontinued the numbers of bacteria in the milk increased, but as the secretion of milk diminished the number of bacteria also decreased. The bacterial content was greater in milk first drawn off than in the last portion. They found that the number of organisms depended on two circumstances:

- (1) The time interval since the last act of suckling.
- (2) The quantity of milk drawn off immediately before the test sample.

The shorter the time the milk was allowed to remain in the breast, the less the number of organisms present in it.

In the milk of 48 women they found *Staphylococcus albus* in pure culture in 36, *S. aureus* in 1, *S. albus* and streptococci in the remainder. In their opinion milk from healthy mammary glands contains cocci, especially *S. albus*, which are present in varying numbers and penetrate from outside into the milk.

Work on this subject has also been done by Honigsmann (1893) who found *S. albus*, and to less extent *S. aureus*, in the milk of healthy women.

Ringel (1893) has investigated the breast-milk from 25 cases. He discarded the first milk withdrawn, and then cleaned the nipple with alcohol and perchloride of mercury. He considers that human milk nearly always contains *S. albus* and less commonly *S. aureus*, and that the organisms penetrate from without into the mammary gland without causing any ill effect.

Before going further we wish to state most emphatically that the results of our investigations do not throw any doubt upon the unquestionable value of breast feeding, which is the only proper method of feeding the infant, but at the same time it is well to realise that infection of the infant from the mother's milk is a greater possibility than has been believed, and that valuable information may be obtained from the bacteriological and cytological examination of the mother's milk and infant's faeces.

#### TECHNIQUE.

Samples of breast milk were collected from 100 different women, the majority taken during the puerperium, but others throughout all stages of lactation. The milk was expressed by hand, taking care not to allow the hand to come into contact with the nipple or surrounding tissues, and was received direct into sterilised bottles. About 2 c.c. of milk were usually taken.

In some cases the nipple was not previously prepared; in others it was cleaned with sterile dry sponges, ether, hydrogen peroxide 10 volumes, and then kept covered for two days with sterile dressings, 2 per cent. iodine in spirit, carbolic lotion 1 in 60, and perchloride of mercury 1 in 1000 and 1 in

2000. In some cases milk was taken in two separate portions from the same breast and the first and second samples examined separately. The majority of the samples of milk were taken from healthy women with normal breasts, but some also from women with pyrexia from various causes, such as puerperal sepsis, and from women with varying degrees of inflammation of the breast culminating in breast abscess. The stools and urine of infants and the urine of mothers were examined when considered necessary.

#### MILK (1).

Each sample of milk was sent to the laboratories in sterile glass-stoppered bottles. On arrival it was well mixed, and 0.025 c.c. withdrawn in a sterile pipette and spread all over the surface of an agar plate and the same amount on litmus lactose agar. Each plate was incubated aerobically at 37° C. for twenty-four hours, the colonies counted, differentiated and subcultured as considered necessary; the plates were then discarded. 0.5 c.c. of milk was added to a tube of 1 per cent. lactose, and glucose, and 1.0 c.c. incubated in a sterile tube at 37° C. for twenty-four hours, when films were made, and if the results obtained appeared to differ from the surface colonies on the plates referred to, then further plating was done and the results compared with the original sample of milk. This method was of undoubted value as instances occurred when streptococci and *B. coli* were cultivated in one or more of the liquid media, although absent on the direct plates. Whenever streptococci were cultivated from a sample of milk, one or more colonies were selected and passed through the usual media, tested for haemolysis, morphological appearances, and in some instances for heat resistance. Staphylococci were tested for evidence of haemolysis, and in many instances titrated with *S. aureus* and *S. albus* anti-sera. All strains of *Bacillus coli* were examined in detail culturally for evidence of haemolysis, and serologically with the colon anti-sera.

The remainder of the milk was centrifugalised and film preparations were made from the deposit and from the cream. It is essential to make films from both sources as although the cells may be equally abundant in each portion, yet, wide variations occur.

The wet films were fixed in Schaudinn's solution and stained with Mayer's haemalum. No counter stain was employed in most instances; occasionally mercurochrome, safranin, or Bordeaux red was used. Perfect film preparations were obtained by this method of fixation, so that cell differentiation was readily obtained, and the usual difficulties experienced by previous workers with cow and human milk, when dried films were employed, were avoided. The cells are readily differentiated and the true polymorphs stand out distinctly.

Total cell counts can be made by the Strong-Seligman method. It may be possible to make the "drops" from the well-mixed undiluted milk, but if the cells are numerous dilution in formalised citrated saline, as employed for total leucocyte counts, is satisfactory. If the drops are made from undiluted milk, it is necessary to fix in the wet in Schaudinn's fixative.

## BABY'S FAECES (2).

If from the bacteriological observations of the mother's milk it was considered necessary to examine the infant's faeces, a sample of the latter was obtained. This was dried by Dudgeon's method (*vide* Wordley, 1921) on unglazed tiles, and the dried powder spread on plates of blood agar, litmus lactose agar, and plain agar, and added to tubes of milk, starch, and Robertson's medium.

Full investigations of the bacteria cultivated from the faeces were made as employed for the bacteria isolated from the milk.

TO SHOW THE DIFFERENCE IN THE ACTION OF BACTERIA ON  
HUMAN AND COW'S MILK.

Four strains of *B. coli* isolated from the urine of cases of pyelo-cystitis, three strains of *B. coli* obtained from infected human milk, and three strains of streptococci isolated from a similar source were cultivated in tubes of 5 c.c. of human and cow's milk, which had been steamed at 100° C. for one hour. In every instance both samples of milk were acidified in forty-eight hours, but at the end of one week's growth at 37° C. each sample of cow's milk was firmly clotted and each tube of human milk was acidified, but no clotting had occurred. The human milk used in these experiments was collected in the usual way from four mothers, and was pooled as each supply was normal. Other samples of human milk were obtained and tested with other strains of *B. coli* and *S. aureus*, but clotting did not occur. Certain strains of *B. proteus* peptonised human milk, but apart from this effect produced by *proteus* bacilli, the only change recorded with all the bacterial strains employed by us has been acid formation, and with cultures of *B. paratyphosus* B. subsequent alkaline formation. This difference between human and cow's milk is distinctive, and it may be a question of practical importance in the feeding of infants when cow's milk is substituted for human milk. In a similar way it is a well-recognised fact that human milk when treated with rennet does not form the firm solid clot such as occurs with cow's milk.

## BACTERIOLOGICAL OBSERVATIONS ON THE MOTHER AND CHILD.

The results of the investigations of the mother and child, referred to in detail below, indicate that the aerobic intestinal flora of the breast-fed infant may be very abnormal, both as regards numbers and strains of the bacteria which are isolated.

In cases 1 and 6 very strong evidence was obtained that the abundant growth of haemolytic colon bacilli in the faeces of the baby was due to infection from the mother's urine.

In case 1, the child was severely ill from a gastro-intestinal infection for many weeks, as already described by us (*vide* Jewesbury and Dudgeon, 1923), and, in our opinion, it cannot be too forcibly emphasised that the new-born infant may be directly infected owing to cystitis in the mother.

Our bacteriological observations described in this report have compelled

us to realise that although some samples of mother's milk actually consist of a mixture of pus and milk heavily infected with *Staphylococcus aureus*, *Bacillus coli* and streptococci, yet no ill effect on the infant may be apparent during the period of observation. We offer the suggestion, however, that the passage of masses of bacteria such as referred to along the intestinal tract of babies for days at a time, or even weeks, may result in the lodgment of these organisms in the tissues, and form a focus of infection at a subsequent period. This view is accepted in the case of cow's milk in relation to the subject of tuberculosis.

We have seen cases of severe boils in breast-fed infants for which no satisfactory explanation was forthcoming, although breast feeding had been replaced by cow's milk because it was believed, for want of a better explanation, that the human milk was too "rich."

The experimental work of Moody and Irons (1923), however, does not lend support to our suggestion; when they introduced by the stomach tube, suspensions of *B. pyocyaneus*, *B. prodigiosus* and *Streptococcus haemolyticus*, they were unable to cultivate these organisms from the blood, chyle or viscera.

To over-estimate the importance of the presence of bacteria in human milk is a very serious error, as in many instances the bacterial flora is without significance however numerous the bacteria may be, and in the vast majority of instances the bacterial flora of human milk is so infinitely better than the best cow's milk, that to offer comparisons would be absurd. It is necessary, however, to call attention to the bacterial defects of human milk, more especially as the literature on this subject is so scanty, and such defects as may occur can be remedied. It is well, therefore, to realise, as we have shown in this communication, that human milk may be heavily infected with pyogenic bacteria, which no cleansing of the nipple and areola will prevent.

Dudgeon (1924), in his Presidential Address to the Section of Tropical Diseases and Parasitology (R.S.M.), in 1924, explained the importance of an accurate knowledge of a patient's diet, if deductions are to be made from an examination of the faeces as to intestinal infection in the human subject.

The investigations of the bacterial flora of infants' faeces have shown that it closely corresponds to the bacterial content of the infant's food, and whenever abnormal findings are obtained from an examination of the faecal flora in children, the diet factor must be fully investigated bacteriologically. It is especially in infants that such investigations can be undertaken most efficiently (cases 1-14).

The constant occurrence of *S. albus* in human milk is readily explained and in the majority of instances is without significance, but it is of interest to record that Todd (1922) showed that this organism was present in 55 per cent. out of 101 examinations of the faeces of infants.

**Case 1. MOTHER. Milk:** Polymorphs +. Abundance of *Streptococci* and some *Staphylococci*. *Streptococcus* is non-haemolytic and forms short chains.

**Urine and Lochia:** Pure *B. coli* which is haemolytic and agglutinates with haemolytic colon anti-serum (Dow).

**BABY.** *Faeces*: Very abundant growth of apparently same *Streptococcus* as in the milk and same haemolytic strain of *B. coli* as in mother's urine.

*Vomit*: Same result as in case of faeces.

*Clinical History*: Baby very ill for several weeks from acute gastro-intestinal infection.

**Case 2. MOTHER.** *Milk*: Polymorphs 46 per cent. *S. albus* + + +.

*Urine*: Pure bacilluria due to *B. coli* (N.H.).

**BABY.** Never breast fed.

*Vomit*: *Streptococci* +, *S. albus* + + +.

*Faeces*: *B. coli* (N.H.). *B. coli* in faeces of baby and urine of mother not apparently same strain.

**Case 3. MOTHER.** *Milk*: Clumps of polymorphs filled with diplococci. *S. aureus* + + + +. (Breast prepared with 1 in 2000 perchloride of Hg.)

**BABY.** *Faeces*: *S. aureus* + + +.

**Case 4. MOTHER.** *R. breast* prepared with ether. Polymorphs 26 per cent. *S. albus* + + +. Liquid media gave *B. coli* in addition to *S. albus*.

*L. breast.* Abundant growth of *B. coli* and *S. albus*.

*B. coli* colonies from right and left breast are not haemolytic and do not agglutinate with the colon anti-sera. All milk *S. albus* colonies agglutinate with *S. albus* anti-serum prepared from case No. 19.

*Urine*: sterile.

**BABY.** *Faeces*: *S. albus* + + +. It agglutinates with *S. albus* anti-serum No. 19.

**Case 5. MOTHER.** *Milk*: Nipple cleaned with ether. No polymorphs. Very feeble growth of *S. albus*.

**BABY.** *Faeces*: *S. albus* +, *B. coli* + + +, *Streptococci* + + +.

**Case 6. MOTHER.** *Milk*: Breast cleaned with ether. No polymorphs. *S. aureus* + + +, *S. albus* +. *S. albus* culture agglutinates with *S. albus* anti-serum No. 19.

*Urine*: Pus and bacilli. Pure culture *B. coli* (H.) which agglutinates with colon anti-serum, haemolytic type (Dow).

**BABY.** *Faeces*: *B. coli* (H.) + + +, *S. albus* +. This haemolytic strain of *B. coli* also agglutinates with same colon anti-serum (Dow).

**Case 7. MOTHER.** *Milk*: *R. breast* prepared with ether. Polymorphs + + +, *S. albus* + + +, *B. coli* +.

*L. breast*, unprepared; no polymorphs. *S. albus* + + +, *B. coli* (N.H.) +.

*Urine*: No *B. coli* obtained.

Milk re-examined fourteen days later. No *B. coli* found, but pure *S. albus*.

**BABY.** *Faeces*: *B. coli* (N.H.) + + +, *S. albus* +. Colon strain inagglutinable with the anti-sera.

**Case 8. MOTHER.** *R. breast* prepared. Polymorphs + + +, *S. albus* + + +, *S. aureus* +, *B. coli* (N.H.) + +, *Streptococci* +.

*L. breast*, unprepared. *S. albus* + + +, *B. coli* (N.H.) + +, *Streptococci* +.

*Urine*: No *B. coli* isolated. Pus and masses of cocci which gave pure growth of *S. albus*.

**BABY.** *Faeces*: *S. albus* + + +, *B. coli* (N.H.) +.

Milk and faecal strains of *B. coli* do not correspond.

**Case 9. MOTHER.** *Milk*: Polymorphs + + +, *Staphylococci* + + +, pure *S. aureus* + + +.

**BABY.** *Faeces*: *S. aureus* + + +, *S. albus* few, *B. coli* + + +.

**Case 10. MOTHER.** *Milk*: Pus present and masses of *Staphylococci*. *S. aureus* + + +, Long chain *Streptococcus* +.

**BABY.** *Faeces*: *S. aureus* + + +, *B. coli* (N.H.) +.

This strain of *B. coli* agglutinates with a colon anti-serum.

**Case 11. MOTHER.** First supply of milk (half an ounce). *S. aureus* + + + +, *S. albus* +. Second supply of milk, *S. aureus* +, *S. albus* + + + +.

**BABY.** *Faeces*: *S. aureus* + + +, *S. albus* + + +, *Streptococci* + +, *B. coli* +.



**Case 12. MOTHER.** *L. breast* cleaned with ether. No polymorphs. *S. albus* + + +, *Streptococci* +.

*R. breast*, same result.

**BABY.** *Faeces*: *S. albus* + + +, *S. aureus* + +, *Streptococci* + +.

**Case 13. MOTHER.** *R. breast*, first supply of milk: very few polymorphs. *S. aureus* + + +. Second supply of milk, after cleaning with iodine: *S. aureus* + + +, *S. albus* +, *Streptococci* few.

*L. breast*, pure *S. aureus*.

**BABY.** *Faeces*: *S. aureus* + + +, *S. albus* + +, *Streptococci* very few.

**Case 14. MOTHER.** *R. breast*, Mastitis. Polymorphs + +, *S. aureus* + + +, and *Streptococci* (N.H.).

Milk obtained just before abscess opened: *S. albus* + +, *S. aureus* +, *Streptococci* + (N.H.).

*L. breast*, "normal." Polymorphs + +, *S. aureus* + + +, *Streptococci* + + + (N.H.). Left breast suppurated fourteen days later.

*Urine*: Sterile.

*Cervical Swab*: *Streptococci* (H.) + + +, *S. albus* + + +, *S. aureus* +.

*Blood culture*: Sterile.

**BABY.** *Faeces*: *S. aureus* and *S. albus*.

H. = Haemolysis. N.H. = No haemolysis. + + +, + +, + signify degrees of bacterial activity.

#### STREPTOCOCCI.

Streptococci were isolated from 49 per cent. of the samples of human milk obtained from our cases. The positive findings, however, among the specimens of normal milk were approximately the same as we obtained from the cases with evidence of inflammation in the breast, although the number of streptococci isolated from some of the samples of milk which showed a high percentage of polymorphs was far greater than occurred with the normal milk. No positive evidence was obtained of a direct relationship between the streptococci isolated from the milks and those obtained from the blood and tissues in puerperal fever.

Streptococci were isolated from 0.025 c.c. of milk plated direct on agar and litmus lactose agar, or in some cases the primary growth occurred only in the tubes of dextrose or lactose to which 0.5 c.c. of milk had been added, or in the 1 c.c. of incubated milk. These strains of streptococci were tested for evidence of haemolysis on human red cells in liquid media by the method employed by Dudgeon and his co-workers (1921), for their morphological characteristics, and the action on lactose, dextrose, salicin, mannitol, inulin and cow's milk. Every strain was gram-positive, and in all but two instances failed to haemolyse human red cells.

There was only a "trace" of haemolysis in the tubes of 0.5 and 0.85 per cent. of sodium chloride in the two positive cultures. The samples of milk from which the haemolytic streptococci were cultivated gave a very low bacterial content, no polymorphs, and nothing else abnormal in the milk, while the mothers' breasts and the infants were healthy. Streptococci with different morphological and cultural reactions were isolated in several instances from the same samples of milk. A method of classifying these streptococci was not obtained from a

study of the morphological and such cultural reactions as referred to, and from haemolytic evidence. For this reason the results are grouped as shown in Table I, as a detailed description of each individual streptococcus is of no practical utility.

Table I.

No. of strains	Haemolytic test		Morphology			Cultural reaction				
	H.	N.H.	Long chain	Short chain	Diplo-cocci	Lac-tose	Man-nite	Salicin	Inulin	Milk
						+ with long chains:				
						28	4	12	4	
						+ with short chains and diplococci:				
						9	5	6	0	
						Total +	37	9	18	4
43	2	41	31	10	2	Total -	6	34	25	39
										Acid 11 Acid and clot 31 1

The great majority of these streptococci formed a granular deposit in those carbohydrate media which were employed for these tests, and the same average of results was obtained with the long and short chain varieties.

#### *BACILLUS COLI.*

In ten cases, strains of *B. coli* were cultivated from samples of human milk, and in four out of the ten polymorphs were abundant in film preparations of the centrifuged milks. In each instance, with one exception, the colon strains were non-haemolytic, but from the milk of this case both haemolytic and non-haemolytic strains were obtained. Positive findings occurred more frequently from samples of 1.0 c.c. of milk incubated in sterile tubes, or in tubes of dextrose and lactose to which 0.5 c.c. of milk had been added, than from milks plated direct. The haemolytic strain was agglutinated with the haemolytic colon anti-serum (Dow), prepared by Dudgeon, Wordley and Bawtree (1922), while only one of the non-haemolytic strains agglutinated with a non-haemolytic colon anti-serum. None of these milks were abnormal in appearance, and did not produce any ill effect on the infants so far as we could ascertain. The cultural reactions of these ten strains were without significance, although 83 per cent. fermented saccharose.

#### THE EFFECT ON THE BACTERIAL CONTENT OF THE MILK FROM CLEANING THE NIPPLE AND AREOLA WITH DRY STERILE WOOL, OR WITH VARIOUS CHEMICAL PREPARATIONS, OR BY "FLUSHING" THE DUCTS WITH MILK.

Observations were made on the bacterial content of the milk obtained direct from the breast without preparation and on subsequent samples after cleaning the nipples and areola, with iodine, ether, perchloride of mercury 1:1000 or 1:2000, or by rubbing with sterile wool, or by "flushing" the ducts with the first milk which was discarded. The breasts were prepared at the time of the examination, or for one or two days previously. A comparison was made with milk obtained from the same side before and after preparation, or from samples taken from the unprepared and prepared breasts, although the

former procedure is the more satisfactory as it is less open to fallacies. Milk collected in the usual manner from prepared and unprepared breasts was plated on agar and litmus lactose agar. It is obvious that cleansing of the skin, as far as it concerns the number of staphylococci, especially *S. albus*, will produce a diminished bacterial growth, but in cases of infection this reduction does not occur, or only to a limited degree. The actual number of colonies of *S. albus* present in milk is generally of no importance, as they are derived from the areola and skin around the openings of the ducts, and partly from the infant. It is now known that this organism is frequently present in the faecal flora of infants (Todd), as referred to in this communication. In cases which show a high polymorph count in the milk, the predominant organisms are *S. aureus*, and to a less extent *S. albus*, streptococci and *B. coli*.

Table II.

The bacterial content of milk obtained from "prepared" and "unprepared" breasts.

(In all these experiments 0.025 c.c. of milk was plated on the surface of the medium.)

A. Both examinations made on same side.

Unprepared					Prepared				
No.	No. of colonies		Pre-dominant organism	Poly-morphs	Method of preparation	No. of colonies		Pre-dominant organism	Poly-morphs
	Agar	L.L. <sup>1</sup> Agar				Agar	L.L. <sup>1</sup> Agar		
1.	1600	480	<i>S. albus</i>	—	"Flushing" ducts	1280	2784	<i>S. albus</i>	—
2.	3000 + <sup>2</sup>	46	"	—	"	3000 +	—	"	—
3.	1860	236	"	—	"	2500	—	"	—
4.	3000 +	1024	<i>S. aureus</i>	—	Iodine	1184	672	<i>S. aureus</i>	—
5.	3000 +	184	<i>S. albus</i>	—	"	—	—	"	—
6.	1168	176	"	—	"	22	7	<i>S. albus</i>	—
7.	—	74	<i>S. aureus</i>	—	Ether	—	93	<i>S. aureus</i>	—
8.	3000 +	—	<i>S. albus</i>	—	"	17	—	<i>S. albus</i>	—
9.	3000 +	—	<i>B. coli</i>	+	"	1700	—	<i>B. coli</i>	+

B. Examinations made from opposite sides.

1.	7	5	<i>S. albus</i>	—	Ether	3000 +	292	<i>S. aureus</i> <i>S. albus</i>	+++
2.	2800	—	"	—	Ether for 2 days	5	—	<i>S. albus</i>	—
3.	3000 +	212	"	—	Iodine				
						3000 +	112	"	+

<sup>1</sup> = litmus lactose.

<sup>2</sup> + = over 3000 colonies present, beyond which number it was impossible to estimate.

CYTOLOGY.

Many observations have been made on the varieties of cells met with in human milk, and discrepancies have occurred as in the case of cow's milk, which is largely due, in our opinion, to the methods employed in the preparation of the films. We have found, as already stated, that the most satisfactory method for the study of the cells met with in human milk is to centri-

fugalise a fresh sample and make film preparations from the deposit and the cream, as it is essential to examine both portions. In most cases the cells appear to be evenly distributed in the deposit and in the cream, but very definite exceptions occurred. Film preparations made from the centrifugalised deposit or from the surface cream were fixed *in the wet* in Schaudinn's solution, and subsequently stained with Mayer's haemalum and counter-stained if necessary. Film preparations which are allowed to dry before they are fixed are useless. Czerny (1890) stated that in his opinion when the breast function is upset leucocytes pass into the milk. He showed in the case of a cat that the lymph glands nearest to the mammary gland were full of the same type of leucocyte as found in the milk.

Berka (1911) from his examination of human milk concluded that the cells are lymphocytes of the large uninuclear variety, but from disintegration become polymorphic. They are capable of absorbing fat and carrying it to the nearest lymph gland.

Film preparations were made from the centrifugalised deposit and from the "cream" obtained from the 100 samples of milk in this series, and total cell counts were made in some instances. One sample of colostrum was examined and film preparations were made and fixed in the wet. Polymorphs were found in large numbers, free and in clumps, while large mononuclear cells filled with small fat droplets, or with one large fat droplet which compressed the nucleus, small mononuclear cells, and free fat droplets were also present.

In 15 cases out of the 100, polymorphs were found in the milk in relatively large numbers, either as individual cells or in small or large clumps. In a few instances differential counts were made, but the difficulties experienced outweighed any possible advantages which might be derived from so laborious a study.

In four instances the percentages of polymorphs in the film preparation of the milks were as follows: 29, 65, 46 and 26 per cent. The presence of polymorphs in large numbers in milk is of practical importance, as, after the fourth day of lactation, it is an indication of inflammation in the breast, although there may not be at the time any clinical evidence in favour of this.

In five cases in this series polymorphs were found in large numbers in the milk, although the breasts were believed to be normal. During the course of the next few days clinical evidence of inflammation was present and finally suppuration occurred.

It would appear, however, from one single examination of colostrum, and from several examinations of "milk" during the first three or four days of lactation, that the presence of numerous polymorphs at this stage is not necessarily an indication of acute inflammation or suppuration. By the 7th or 8th day and later, normal human milk shows extremely few cells, and such cells as are present are degenerated epithelial cells with a few or abundance of fat droplets. Small mononuclear cells may be found, but polymorphs are so scarce as to be a negligible quantity.

In two cases polymorphs were present in large numbers in film preparations of milk, lying singly and in large clumps together with masses of cocci. Pure cultures of *S. aureus* were obtained from one case, and *S. aureus* and streptococci from the other, and in both cases suppurative mastitis developed.

The bacteriological findings in the sixteen cases which showed numerous polymorphs in the film preparations of milks are of sufficient importance to refer to at some length, as the results confirm our view that the presence of large numbers of polymorphs in milk, quite apart from the clinical condition of the patients, is of considerable significance; in fact, the presence of numerous polymorphs in human milk after the 4th day of lactation is an indication to keep careful observation on the mother's breasts for an acute inflammatory complication. The very large number of colonies on agar plates in 13 cases out of 16 exceeding the maximum limit of 3000 colonies from 0.025 c.c. of milk, which could be accounted for accurately, is far in excess of the average number of colonies obtained from normal cases or cases without a polymorph increase. The high percentage of *S. aureus* cases is also a distinctive feature. The strains of *S. aureus* obtained from milk were haemolytic on blood agar. Many strains grown on agar slopes were very difficult to emulsify in saline for agglutination tests, even when repeated subcultures on agar were made.

Table III.

No.	No. of colonies on agar from 0.025 c.c. of milk	Bacteria present in order of frequency	Clinical course	
			At time	Subsequent
1.	84	<i>S. albus</i> , streptococci	nil	—
2.	3000	<i>S. aureus</i> , streptococci	"	—
3.	3000	<i>S. albus</i> , <i>S. aureus</i> , and streptococci	"	—
4.	3000	<i>S. albus</i> , <i>S. aureus</i>	Mastitis	Abscess
5.	3000 <sup>1</sup>	<i>S. aureus</i> , streptococci <sup>2</sup>	—	—
6.	1700	<i>S. albus</i> , <i>B. coli</i>	nil	—
7.	3000	<i>S. aureus</i> , <i>S. albus</i>	"	—
8.	3000	<i>S. albus</i>	—	—
9.	3000	<i>S. albus</i>	—	—
10.	3000	<i>S. albus</i> , <i>S. aureus</i> , <i>B. coli</i>	Mastitis	—
11.	3000	<i>S. aureus</i> , <i>S. albus</i> , streptococci	nil	—
12.	3000	<i>S. aureus</i> , streptococci <sup>2</sup>	Mastitis	Abscess
13.	3000	<i>S. aureus</i> <sup>2</sup>	"	"
14.	3000	<i>S. aureus</i>	"	"
15.	3000	<i>S. aureus</i> , <i>S. albus</i>	nil	—
16.	492	<i>S. albus</i>	"	—

<sup>1</sup> Nipple prepared for 2 days with 1 in 2000 perchloride of mercury.

<sup>2</sup> Cocci present in film preparations of the milk.

#### CONCLUSIONS.

(1) The cleansing of the nipple and areola, before samples of milk are withdrawn, with chemicals such as iodine and ether may effect a considerable reduction in the bacterial content of normal milk.

(2) If a high bacterial content is associated with numerous polymorphs in the milk, cleansing of the nipple with chemicals or flushing of the ducts will not greatly reduce the number of bacteria.

(3) The most important organism in samples of milks from cases of mastitis, suppurative mastitis, and those in which polymorphs are numerous, is the *Staphylococcus aureus*.

(4) Streptococci were isolated from 49 per cent. of the samples of human milks. Only 2 per cent. of these streptococci were haemolytic. There is no evidence that the presence of streptococci in human milk is an indication of a pathological process in the breast, although the number of streptococci present in milks with a high polymorph count was greater than in the normal milk. The haemolytic streptococci were not associated with any apparent abnormal condition in mothers or infants.

(5) *B. coli* strains were isolated from ten cases. In nine instances the organisms were non-haemolytic and in one haemolytic. The haemolytic strain reacted with a haemolytic colon anti-serum. In four out of the ten cases polymorphs were abundant in the milk. There was no evidence of ill health in the infants fed on these milks.

(6) Normal human milk may contain *S. albus*, *S. aureus*, streptococci or *B. coli*. The commonest organism is *S. albus* which may be associated with one or more of the bacteria referred to.

(7) The cytological examination of fresh human milk must be made a routine procedure in all cases if a bacteriological investigation is necessary. We recommend centrifugalisation of the milk and the fixation of the *wet films* prepared from the deposit and the cream in Schaudinn's solution. After the 4th day of lactation, polymorphs are relatively scarce in normal milk, but if found to be numerous, lying separately and in clumps, the mother's breasts should be carefully watched for clinical evidence of inflammation and the milk should be examined bacteriologically. The presence of numerous polymorphs in a sample of fresh milk obtained after the 4th day of lactation, is an indication of infection which may not be associated with any clinical manifestations although may subsequently give rise to mastitis.

(8) Actual pus may be present in milk, before there is clinical evidence of mastitis.

(9) The cultures of *S. aureus*, *B. coli* and streptococci employed by us failed to clot human milk.

(10) The bacteriological flora of an infant's faeces may serve as a definite indication of the bacterial content of the mother's milk.

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# THE INFLUENCE OF CARBOHYDRATES ON HYDROGEN SULPHIDE PRODUCTION BY *BACILLUS AERTRYCKE* (MUTTON).

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(With 4 Charts.)

AN important aspect of the investigation of bacterial metabolism is the study of the substrate and its changes and of the resulting products; together with an examination of the rate of change. The present report deals with the influence of certain carbohydrates in modifying the action of one species of bacteria upon the protein molecule.

Hirschler (1886) found that sugars inhibited the production of indol and other phenolic bodies, and preferred the explanation that sugars were more easily decomposed than proteins. He showed that in mixed cultures proteins are not attacked when sugars are present, although the acids formed were neutralised by calcium carbonate.

Smith and also Peckham (1897) concluded that non-production of indol in sugar media was due to acid formation. This seemed to be supported by the work of Glenn (1911), who found that 0.5 per cent. of added lactic acid prevented indol formation, and also that the presence of sugars prevented the liquefaction of gelatine. It has, indeed, frequently been stated that organisms growing on a sugar-protein medium do not form indol, liquefy gelatine nor digest casein, and that ammonia production is greatly lessened. Acid production from sugars has been put forward as an explanation for this inhibition of the production of protein decomposition products.

Fischer (1915) found that neither hydron concentration nor total acidity influenced indol production; and suggested that glucose inhibited the action of the proteolytic enzyme.

Kendall, Day and Walker (1913) adopted a very similar hypothesis, in stating that "protein sparing" is not due to acids produced from sugars, but that sugars serve the energy requirements more readily than do proteins, and that the activity of the organisms is diverted from the protein to the sugar, so that protein decomposition products are found wanting. They state that the slight ammonia production, which occurs, indicates the small amount of protein decomposition necessary for structural requirements. It is generally stated that the production of acid from sugar inhibits the liquefaction of gelatine by *B. proteus*, but Kendall and Walker (1915) found that enzymes from this organism reacted in the presence of glucose or of organic acids as



easily as in their absence. In dextrose gelatine, however, no proteolytic ferment was formed until the dextrose had been completely utilised.

Jordan (1917) states that the hydron concentration of the medium is not an explanation of the protein sparing effect, and is only of importance in so far as it influences the general conditions of growth.

Logie (1919) found that the production of indol was decreased in the presence of sugars, and increased in amount in the absence of sugars, and suggested that this was due to the increased utilisation of indol in the presence of sugars.

Jones (1916) found that, with varying amounts of glucose, *B. proteus* produced proteolytic enzymes first in the cultures containing the smaller amounts of carbohydrate. He believed that the absence of protein-splitting in sugar media was due to the fact that the proteolytic ferment does not appear till the utilisable sugar has undergone complete hydrolysis, or that the ferment is unable to act in an acid medium.

Berman and Rettger (1918) found that the presence of sugar did not affect the nitrogen metabolism, the concentration of the amino nitrogen remaining unchanged both in plain, and in glucose media inoculated with *B. coli*.

De Bord (1923) found that the total amount of amino nitrogen may be increased to but a slight extent during bacterial growth in plain medium of peptone free from glucose, while in the presence of glucose a marked increase in amino nitrogen takes place, using Folin's amino nitrogen method and not Sørensen's method of estimation. He found the production of amino nitrogen was always greater in the presence of glucose, but was unable to correlate the amino nitrogen figures with the number of living cells. His explanation was that the amino nitrogen in plain media was used as rapidly as formed, but that in sugar media the production is in excess of the amounts being used, so that the concentration of amino nitrogen in the culture is increased. He suggests also that the presence of sugar may increase the availability of nitrogen products other than amino nitrogen. A material increase in amino nitrogen he regards as an index of proteolysis, clearly shown in sugar media, while in non-sugar media the amino nitrogen cannot serve as an index of proteolysis, except in those cases in which a definite increase occurs.

In De Bord's experiments the presence of a sugar resulted in lessened ammonia production, and this has been claimed as being due to a decreased proteolysis. Gordon (1917) found that *B. coli* utilised nitrogen from ammonium salts when a sugar was present. It seems, therefore, that decreased ammonia production does not necessarily indicate lessened proteolysis. It may indicate greater ammonia utilisation.

So far most attempts at the elucidation of this problem have been made by a consideration of the fate of the nitrogen complex in the protein molecule. Our attempt has been directed to the sulphur containing fraction of the protein molecule, and with results of no little interest, if not at the moment capable of easy explanation.

We have attempted to estimate the extent of proteolysis by measuring the hydrogen sulphide formation, and to show how this is influenced by the presence of sugars and phosphates. Our attention has been confined to one organism, a known hydrogen sulphide producer—*B. aertrycke* (Mutton).

Previously hydrogen sulphide production has been detected by the blackening of lead solution either in the medium, or on papers suspended over it, and the amount of blackening has been used as an index of the amount of sulphide. This method has been used for various purposes such as:

The differentiation of *Bacillus paratyphosus* A and *paratyphosus* B (Burnet and Weissenbach, 1915), and for the measurement of the pollution of water, Schardinger (1894) and Dunham (1879).

Sasaki and Otsuka (1912) added various sulphur compounds to Fränkel's artificial medium, and found that the majority of the strains of bacteria they investigated produced hydrogen sulphide from cystine and from sulphur, a few from sodium thiosulphate, one or two from sodium sulphite, and none from sodium sulphate, or from taurine. These results were substantiated by the findings of Myers (1920) and Tanner (1917) who also stated that no hydrogen sulphide was produced from 2-thiohydantoin or magnesium sulphate.

Seiffert (1909) investigated the effect of sugars on hydrogen sulphide production. Working with strains of the *Salmonella* group he found that this substance was evolved in very varying amounts, and that the addition of sugar to the peptone water greatly increased the production, as judged by the blackening of lead acetate paper. He used 0.5 per cent. sugar in the media, and sterilised it by steaming for one hour. This may account for some of his results, as the higher sugars would probably be hydrolysed to some extent, and the lower ones possibly destroyed. He found that hydrogen sulphide production was greatest for cane sugar, less for laevulose and galactose, while after twenty-four hours' incubation in the presence of dextrose there was no hydrogen sulphide, and only traces in the presence of lactose.

Myers (1920) found no marked, nor constant effect, with the different carbohydrates he employed, though he refuted Seiffert's results for dextrose and lactose, which sugars he found to have no inhibitory influence on hydrogen sulphide production.

Tilly (1923) observed a curious correlation between sulphide production in lead-acetate agar, and ability to grow in synthetic medium composed of 0.2 per cent. sodium ammonium hydrogen phosphate and 1 per cent. glucose. Out of more than 150 strains of *B. suispestifer* examined, only six failed to conform to the rule that strains which produced hydrogen sulphide also grew on the synthetic medium and *vice versa*.

Wilson (1923) has suggested that the hydrogen sulphide produced during the growth of bacteria in peptone water is derived, at least in part, from sulphites, present as an impurity, and accounts in this way for the very varying results obtained with different brands of commercial peptone.

## EXPERIMENTAL.

1. To ensure that all the sulphur present in the Witte peptone media used was present as organic sulphur, an attempt was made to detect sulphite in the peptone water by means of Votoček's method (1907).

The effect of adding 5, 10 and 15 drops of a 0.00025 per cent. solution of fuchsin to water, and to an equal volume (10 c.c.) of 3 per cent. peptone water was compared. The peptone water did not decolourise the fuchsin, thus indicating the probable absence of sulphite in this particular Witte peptone.

2. In order to obtain a roughly quantitative idea of the amounts of hydrogen sulphide, produced by *B. aertrycke* (Mutton) in peptone water under various conditions, a series of tube experiments was carried out in which an expression of gas production was estimated by measuring the blackening produced on lead acetate paper suspended in a narrow tube, which passed into the experimental tube through a waxed cork. The medium employed was the basal medium used throughout the whole series of experiments, and consisted of 3 per cent. Witte peptone dissolved by steaming in a watery 0.25 per cent. solution of sodium chloride. This was filtered, its reaction adjusted to pH 7.6 and then autoclaved at 115° C. for 20 minutes. 2 per cent. of glucose (sterilised by steaming as a 50 per cent. solution in water) was added to half the tubes, and control tubes were prepared containing varying amounts of iodine, in order to further eliminate the possibility of sulphites in the media. The tubes were inoculated with one loopful of the standard inoculum, used throughout the experiments. This consisted of a four hours' growth of *B. aertrycke* (Mutton) in 5 c.c. of 1 per cent. peptone water, which in its turn had been inoculated with one standard loopful from a stock culture. The tubes were incubated at 37° C., and the hydrogen sulphide produced estimated by measurement of the blackening of lead papers.

Iodine proved to have no effect on the hydrogen sulphide production provided it was not present in a concentration high enough to inhibit growth, thus indicating that the peptone itself was the only source of sulphur.

The results obtained in the glucose and non-glucose media are shown in Table I.

Table I. *Hydrogen sulphide production by B. aertrycke (Mutton) in the presence and absence of 2 per cent. of glucose; as measured by the blackening of lead acetate paper.*

Period of growth	Length in mm. of lead acetate paper blackened	
	In the absence of glucose	In the presence of 2 % glucose
24 hours	0.5 mm.	11.0 mm.
48	2.3	12.7
72	3.0	12.7
96	4.0	12.7
120	5.0	12.7
144	5.0	12.7
240	5.0	12.7

The fact that glucose appeared to accelerate hydrogen sulphide production, led us to devise a more accurate method for its estimation.

250 c.c. of medium was employed, contained in a special flask with a ground glass stopper, through which two tubes passed in such a way that gas could be bubbled through the culture at the end of a period of growth, in order to drive off the hydrogen sulphide produced. This was connected with a series of four Dreschel towers, containing alternately standard iodine solutions and dilute starch paste solutions, the latter to catch any iodine that might be blown over. In a large number of experiments, a fifth tower containing standard sodium thiosulphate solution proved that the amount of iodine blown over further than the fourth tower was negligible.

A stream of carbon dioxide (washed by passing through lead acetate, potash, and water bulbs) was used to distil off the hydrogen sulphide, which in the majority of experiments was small enough in quantity to be entirely oxidised in the first tower, the three subsequent towers serving as controls.

After one hour's exposure to the stream of carbon dioxide, the iodine in the towers was titrated against a standard solution of sodium thiosulphate, and from the figures obtained the volume of hydrogen sulphide produced in certain definite times could be calculated.

The results throughout this paper are expressed in terms of  $N/1000$  hydrogen sulphide, though experiment proved that  $N/100$  iodine solutions were more convenient for use.

Many control experiments were carried out with uninoculated media, and these showed that no iodine of any significance was lost during the gas distillation.

During the early part of the work the medium, after growth and gas distillation, was tested for residual hydrogen sulphide by heat distillation with and without the addition of acid, and examination of the gaseous distillates. Hydrolysis, however, took place to such an extent as to render the figures useless for our purpose, and this method was discarded.

In many experiments 0.42 per cent. of disodium hydrogen phosphate was added to buffer the medium. Experiment proved, however, that the buffering effect of the peptone itself, in the high concentration employed, rendered the effect of added phosphate almost negligible. Nevertheless the results of the phosphate experiments are included here, as they serve to confirm the plain peptone experiments; though, on the whole, the figures obtained tend to be slightly higher.

The second set of experiments consists of a series of quantitative determinations of hydrogen sulphide after various periods of growth. Each figure obtained represents the hydrogen sulphide in one 250 c.c. culture after the stated period of growth, except the figures marked (*a*) which represent the average of the two figures and those marked (*b*) which represent the average of three.

1 c.c. of the standard inoculum was employed, and a parallel series of

cultures was grown in the incubator, and from this a record was obtained each day of the hydrogen-ion concentration of the media during growth.

The results obtained are summarised in Table II, and are shown graphically in Chart I.

Table II. *Hydrogen sulphide production by B. aertrycke (Mutton) in the presence and absence of 2 per cent. of glucose and 0.42 per cent. of phosphate.*

3 % peptone (Witte) in 0.25 % sodium chloride solution together with

Period of growth Hours	No addition		2 % glucose		0.42 % phosphate		2 % glucose + 0.42 % phosphate	
	c.c. $N/1000$ $H_2S$	Hydri- on con- cen- tration	c.c. $N/1000$ $H_2S$	Hydri- on con- cen- tration	c.c. $N/1000$ $H_2S$	Hydri- on con- cen- tration	c.c. $N/1000$ $H_2S$	Hydri- on con- cen- tration
4	—	—	—	—	—	—	10.5	7.5
6	—	—	—	—	—	—	15.1	7.4
8	—	—	—	—	—	—	19.6	7.35
14	—	—	—	—	—	—	177.5	5.05
15	—	—	—	—	—	—	178.0	5.0
16	—	—	—	—	—	—	171.4	4.95
18	27.1	7.3	170.5	5.1	27.3 (a)	7.5	173.5 (b)	4.4
24	—	—	—	—	—	—	—	—
42	34.4 (a)	7.25	215.5	4.8	27.3	7.6	236.5	4.8
48	—	—	—	—	—	—	—	—
66	36.7	7.35	131.7	4.7	—	—	232.0	4.75
72	—	—	—	—	31.7	7.8	—	—
90	42.3	7.4	123.7	4.7	—	—	196.0	4.7
96	—	—	—	—	41.5	7.8	—	—
114	48.9	—	—	—	—	—	145.0	4.7
120	—	—	—	—	57.7	7.9	—	—
138	—	—	100.0	4.7	—	—	—	—
186	—	—	—	—	85.0	8.0	—	—
234	177.3 (a)	8.0	—	—	—	—	—	—
240	—	—	—	—	112.5 (b)	8.0	—	—
258	—	—	112.2	4.7	—	—	—	—
282	115.2	8.0	—	—	—	—	123.3	4.7
345	—	—	101.1	4.7	—	—	—	—

These results seem to show that glucose definitely stimulates hydrogen sulphide production by *B. aertrycke* (Mutton), and it appeared of interest to investigate the effect of varying the concentration of glucose in the media.

A series of lead-acetate paper trial tubes was set up, and to the basal phosphate medium the following amounts of glucose were added: 0.1, 0.25, 0.5, 0.75, 1.0, 1.1, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 4.0, 5.0 per cent.

The areas of lead-acetate paper blackened were practically identical in all of the tubes after 18 hours, and again on the second day of growth, after which no more blackening took place.

Using the quantitative method of hydrogen sulphide estimation, 18 hours was the period of growth chosen to demonstrate stimulation of the hydrogen sulphide production, and amounts of glucose varying from 2 per cent. to 0.0001 per cent. were added to the phosphate medium with the results shown in Table III and Chart II. In this experiment the titrateable acidity of the media, at the end of the period of growth, and after the distillation, was determined by neutralising with caustic soda, to the neutral point of phenolphthalein.

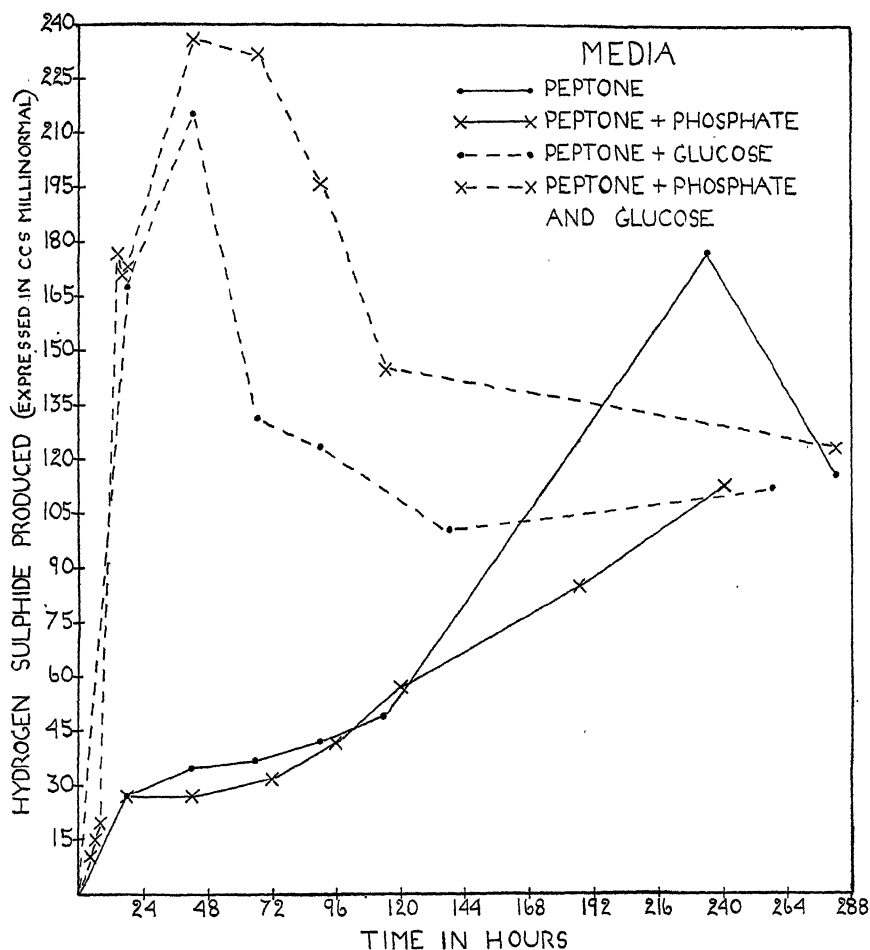


Chart I. Hydrogen sulphide production by *B. aertrycke* (M.) in the presence and absence of 2 % of glucose.

Table III. *Hydrogen sulphide produced in 18 hours by B. aertrycke (Mutton) in the presence of various amounts of glucose.*

% of glucose	c.c. N/1000 H <sub>2</sub> S produced	Vol. N. NaOH to neutralise 250 c.c. media after CO <sub>2</sub> dist.
2.0	173.5 (b)	20.0
1.5	179.0	—
1.0	159.0	19.6
0.5	177.0	—
0.25	142.0	16.3
0.1	173.9 (a)	14.5
0.05	173.5	13.8
0.025	173.7	13.2
0.01	64.25	13.9
0.001	34.46	13.8
0.0001	29.3	12.0
0	29.3	11.8

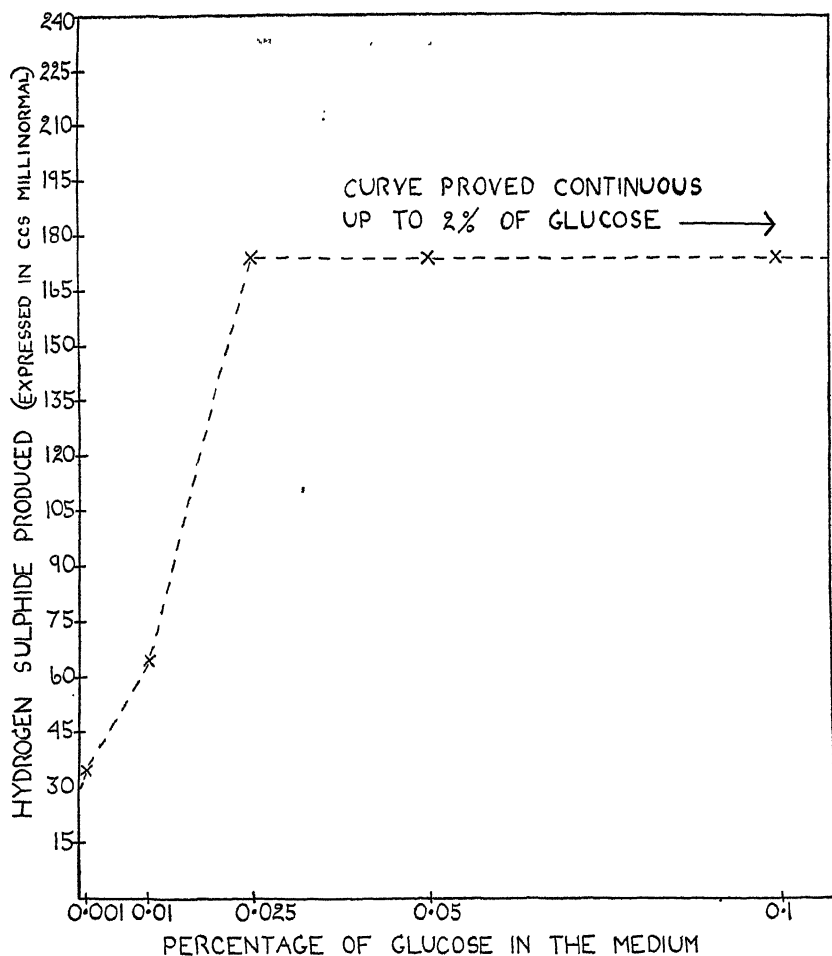


Chart II. Hydrogen sulphide produced in 18 hours by *B. aertrycke* (M.) in the presence of various amounts of glucose.

It will be seen that 0.025 per cent. of glucose proved to be sufficient to produce a maximum acceleration of the hydrogen sulphide production.

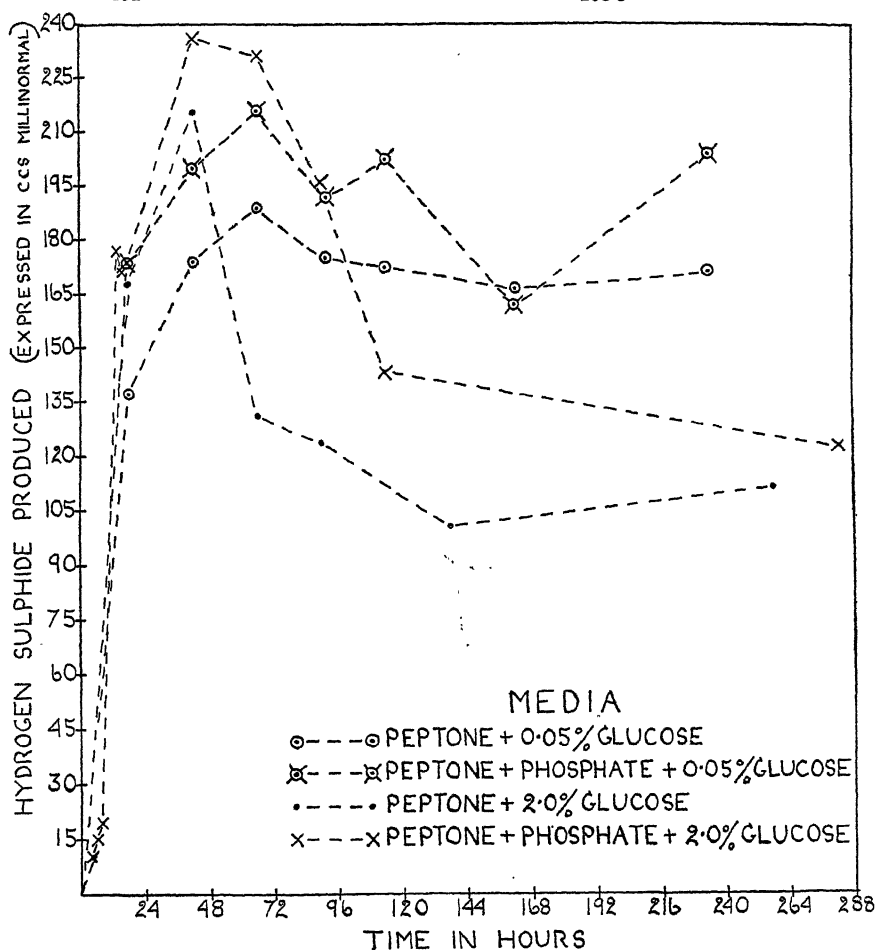
On turning to the literature we found a publication by Ayers and Mudge (1922) who, working on Vitamines, found that the stimulating effect of adding cabbage extracts to cultures of a streptococcus was due to the minute amounts of glucose present in it. They found 0.014 per cent. of glucose to have a stimulating effect on the growth (as measured by turbidity) of their streptococcus in Difco peptone medium.

Our last results led to two series of longer growth experiments using 0.05 per cent. glucose in the plain and phosphated peptone media.

A few experiments were gas-distilled with nitrogen instead of carbon dioxide, but this did not appear to affect the results, which are set out in Table IV and Chart III.

Table IV. *Production of hydrogen sulphide by B. aertrycke (Mutton) in peptone solution containing 0.05 per cent. of glucose with and without added disodium hydrogen phosphate.*

Period of growth Hours	3 % peptone in 0.25 % NaCl solution together with			
	0.05 % glucose		0.05 % glucose + 0.42 % phosphate	
	c.c. N/1000 H <sub>2</sub> S	Hydron concentration	c.c. N/1000 H <sub>2</sub> S	Hydron concentration
18	137.2	6.8	173.5	7.0
42	174.3	—	200.0	—
66	189.0	8.2	215.8	7.6
90	175.2	8.4	191.8	7.9
114	172.5	8.3	202.6	8.0
162	166.5	8.6	162.4	8.2
234	170.7	8.6	203.6	8.2
Nitrogen distilled experiments				
18	—	—	201.8	—
162	—	—	167.0	—
234	—	—	238.5	—

Chart III. The effect of 0.05 % and 2.0 % glucose on the hydrogen sulphide produced by *B. aertrycke* (M.) ± 0.42 % phosphate.



From the later results in this experiment, which are high in H<sub>2</sub>S value in cultures containing small amounts of glucose, it would appear that the disappearance of some of the free hydrogen sulphide from the longer 2 per cent. glucose cultures was due to the presence of comparatively large amounts of glucose decomposition products.

The early parts of the glucose curves being the most interesting, they were investigated by means of continuous gas distillation with nitrogen, as a continuous stream of CO<sub>2</sub> through the medium would have rendered it too acid for the growth of *B. aertrycke* (Mutton). The outlet of the culture flask was connected to a two-way tap so that the gases could be diverted to either of two series of iodine and starch towers, so that at intervals of the distillation, the towers could be renewed without interference with the gas flow.

The results obtained, using the 2 per cent. glucose + 0.42 per cent. phosphate medium, are shown in Table V.

Table V. *Hydrogen sulphide production by B. aertrycke (Mutton) in the presence of 2 per cent. of glucose, during continuous gas distillation.*

Period of growth		N/1000 H <sub>2</sub> S c.c.	Rate of H <sub>2</sub> S production per hour
6 hours		11.3	1.88
Between	6th and 12th hours	226.5	37.75
"	12th and 18th hours	100.0	16.67
"	18th and 24th hours	32.8	5.47
"	24th and 48th hours	17.2	0.96

To eliminate the possibility that the increased hydrogen sulphide production in the presence of glucose is due to the formation of acid and its action on the peptone, we carried out two short series of experiments. One of these consisted in allowing growth to take place in the glucose media in the presence of limestone, so that the hydrogen-ion concentration of the culture was never below pH 7.2. The hydrogen sulphide formed in all cases reached its normal high figure in the usual time.

The other series of experiments consisted in incubating uninoculated media in the presence of varying amounts of acid. In no case did gas distillation reveal the presence of any hydrogen sulphide.

Working with a synthetic medium composed of:

Sodium chloride	...	...	5 grms.
Disodium hydrogen phosphate	...	...	4.2
Sodium citrate	...	...	6
N caustic soda to adjust reaction to pH	...	...	7.6
Distilled water	...	...	1000
Cystine	...	...	0.2

we found that glucose exerted a similar influence to that which it does in a glucose peptone medium.

In order to ascertain the number of viable organisms at different periods of growth, counts were made according to the method of G. S. Wilson (1922), the dilutions being made with calibrated dropping pipettes, and the colonies

counted in roll-tubes. Table VI gives the results of a typical experiment, with the numbers and also the logarithms of the numbers of viable organisms in one c.c. of 3 per cent. peptone water + 0.25 per cent. sodium chloride. In 2 per cent. glucose media the viable count increases rapidly during the first 16 hours, whereas in non-glucose media the count steadily increases, but does not attain to such a high figure even in prolonged periods of growth.

Table VI. *Viable counts of B. aertrycke (Mutton) in the presence and absence of 2 per cent. glucose and 0.42 per cent. phosphate.*

Period of growth Hours	3 % peptone (Witte) in 0.25 % sodium chloride solution together with							
	No addition		2 % glucose		0.42 % phosphate		2 % glucose + 0.42 % phosphate	
	Viable count per c.c.		Viable count per c.c.		Viable count per c.c.		Viable count per c.c.	
	Actual	Log.	Actual	Log.	Actual	Log.	Actual	Log.
4	17,700	4.25	25,550	4.41	18,900	4.28	34,650	4.54
8	8,800,000	6.94	120,500,000	8.08	3,170,000	6.50	120,500,000	8.08
12	51,000,000	7.71	213,000,000	8.33	65,000,000	7.81	194,000,000	8.29
16	51,000,000	7.71	377,750,000	8.58	87,750,000	7.94	397,000,000	8.60
20	84,000,000	7.92	251,500,000	8.40	96,500,000	7.98	369,500,000	8.57
24	86,500,000	7.93	78,000,000	7.89	71,500,000	7.85	85,500,000	7.93
42	112,000,000	8.05	46,000,000	7.66	137,000,000	8.14	1,160	3.06
66	185,000,000	8.27	10,250	4.01	121,500,000	8.08	—	—
90	140,000,000	8.15	3,290	3.52	87,000,000	7.94	—	—
114	143,000,000	8.16	438	2.64	148,000,000	8.17	—	—
138	158,500,000	8.20	—	—	149,500,000	8.17	—	—

On varying the amounts of glucose as follows:

nil, 0.025 per cent., 0.05 per cent. and 2.0 per cent.

we found that 0.05 per cent. gave a maximum viable count in 66 hours, which slowly decreased. 0.025 per cent. glucose seemed to have very little effect on the count, despite the fact that it had a pronounced effect on the hydrogen sulphide production (see Table VII and Chart IV).

This suggests that the glucose is serving a purpose other than that of a source of energy.

These results led to a series of confirmatory experiments in which viable counts and hydrogen sulphide estimations were carried out on parallel cultures incubated together (see Table VIII).

Finally we made a series of amino-acid determinations by the Sørensen method of formol titration, and found, as previous workers have, that in the presence of 2 per cent. glucose, the amino-acid nitrogen figure thus obtained remained unchanged during growth. However, the presence of 0.025 per cent. glucose in the cultures only appeared to keep the figure stationary during the first 66 hours, after which time it began to slowly increase, just as it did during the whole growth period in peptone containing no glucose (see Table IX).

Having summarised the reaction of *B. aertrycke* (Mutton) in the presence of glucose, we next investigated its reaction with various other sugars; maltose, which is the only disaccharide fermented by it; xylose, which may be taken as typical of the three pentoses fermented by it; and sucrose, which it

Table VII. *Viable counts of B. aertrycke (Mutton) in the presence of various amounts of glucose.*

Period of growth Hours	3 % peptone (Witte) in 0.25 % sodium chloride solution together with							
	No addition		0.025 % glucose		0.05 % glucose		2.0 % glucose	
	Viable count per c.c.		Viable count per c.c.		Viable count per c.c.		Viable count per c.c.	
	Actual	Log.	Actual	Log.	Actual	Log.	Actual	Log.
16	—	—	—	—	—	—	377,750,000	8.58
18	46,480,000	7.67	58,000,000	7.76	217,500,000	8.34	279,000,000	8.45
20	84,000,000	7.92	—	—	—	—	251,000,000	8.40
42	112,000,000	8.05	78,000,000	7.89	280,500,000	8.45	46,000,000	7.66
66	185,000,000	8.27	131,500,000	8.12	295,000,000	8.47	10,250	4.01
90	140,000,000	8.15	—	—	—	—	3,290	3.52
114	143,000,000	8.16	149,000,000	8.17	140,500,000	8.15	438	2.64
138	158,500,000	8.20	—	—	—	—	—	—
186	—	—	137,000,000	8.14	52,500,000	7.72	—	—

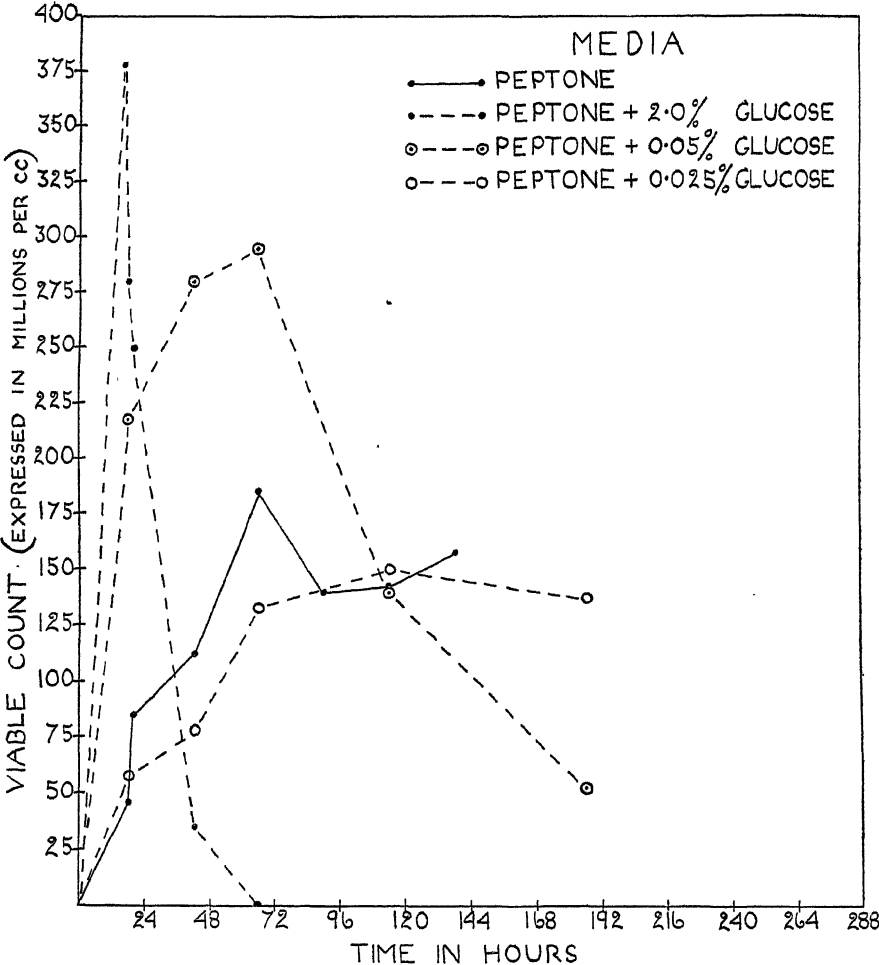


Chart IV. *Viable counts of B. aertrycke (M.) in the presence of various amounts of glucose.*

Table VIII. *Viable counts of, and hydrogen sulphide production by, B. aertrycke (Mutton); in 18 hours in the presence of various amounts of glucose.*

Medium	18 hours' growth	
	Viable count per c.c.	c.c. N/1000 H <sub>2</sub> S
3 % peptone (Witte) in 0.25 % NaCl solution together with		
No addition	32,000,000*	—
	46,480,000	—
	55,000,000	27.1
0.42 % phosphate	32,000,000*	—
	55,000,000	—
	61,500,000	27.3
0.025 % glucose	32,000,000*	—
	58,000,000	—
	62,000,000	144.4
0.025 % glucose + 0.42 % phosphate	67,000,000*	162.5
	71,500,000	105.6
	73,000,000	164.7
0.05 % glucose	217,500,000	137.2
0.05 % glucose + 0.42 % phosphate	369,500,000	173.5
2.0 % glucose	279,000,000	170.5
2.0 % glucose + 0.42 % phosphate	322,600,000	173.5

\* Incubated at 31° C.

Table IX. *Amino-acid nitrogen determinations in cultures of B. aertrycke (Mutton) containing no added glucose, 2 per cent. glucose, and 0.025 per cent. glucose.*

Period of growth Hours	3 % peptone (Witte) in 0.25 % sodium chloride solution together with		
	2 % glucose +		
	0.42 % phosphate	0.42 % phosphate	0.025 % glucose
	Amino-acid nitrogen	Amino-acid nitrogen	Amino-acid nitrogen
	mgm. per 250 c.c.	mgm. per 250 c.c.	mgm. per 250 c.c.
0	70.0	70.0	78.75
18	74.38	70.0	78.75
42	78.75	70.0	74.38
66	78.75	52.5	78.75
138	96.25	70.0	96.25
210	113.75	70.0	113.75
402	157.50	70.0	148.75

does not ferment. We were compelled to add the sugars in the dry state without sterilisation, as the trace of glucose inevitably produced by sterilising would have been sufficient to cloak all other reactions.

The large amount of hydrogen sulphide produced by cultures of *B. aertrycke* (Mutton) in 3 per cent. peptone water to which varying quantities of maltose had been added soon convinced us that the reaction in the presence of maltose was similar to the glucose reaction, though possibly the acceleration was slightly greater (see Table X).

Table X. *Hydrogen sulphide production by B. aertrycke (Mutton) in 18 hours in the presence of various percentages of maltose.*

% maltose	c.c. N/1000 H <sub>2</sub> S
2.0	229.3
0.05	258.6
0.25	166.2
0.01	59.6

With xylose the reaction proved slightly different, 2 per cent. of xylose gave a fairly high figure in 18 hours, but 0.05 per cent. and 0.1 per cent. showed a lag period, during which the xylose appeared to have no influence on the hydrogen sulphide production, and it was not until between the 42nd and 66th hours of growth that the hydrogen sulphide production appeared to be accelerated (see Table XI).

Table XI. *Hydrogen sulphide production by B. aertrycke (Mutton) in the presence of various percentages of xylose.*

Period of growth	0.05 %	0.1 %	2 %
18 hours	20.5 c.c.	23.23 c.c.	115 c.c.
42	38.0	27.0	
66	178.0	181.8	
234	129.6	238.7	

With sucrose we experienced great difficulty in obtaining a sample absolutely free from glucose.

We found that laboratory "pure" sucrose gave a maximum acceleration of the hydrogen sulphide production, though the amount of acid produced in the cultures was not sufficient to be detected by indication with Andrade reagent or phenol red; the hydrogen-ion concentration by colour comparison being the same as those of cultures containing no added sucrose.

The results led us to believe that the acceleration was due to the presence of traces of glucose, and to eliminate this we endeavoured to prepare pure sucrose by recrystallisation from absolute alcohol, finally obtaining a sample which did not accelerate the hydrogen sulphide production, though after a prolonged period it rose to a higher figure than we had obtained in the absence of added sugar (possibly this was due to the slow hydrolysis of the sucrose in alkaline solution) (see Table XII).

Table XII. *Hydrogen sulphide production by B. aertrycke (Mutton) in the presence of 2 per cent. pure sucrose.*

Period of growth	c.c. $N/1000 H_2S$
18 hours	12.0
42	23.5
66	47.0
234	202.0
354	235.0

#### DISCUSSION.

The results obtained show that there is a considerable degree of parallelism between the influence of carbohydrates on bacterial multiplication and their influence on the production of hydrogen sulphide.

In peptone media, without glucose, bacterial multiplication passes through its well-known phases, and is succeeded by a balance between the generation-time and death-rate of bacteria, such that the viable count is maintained at a high figure over several days. This phase of equilibrium gradually gives place

to a stage in which the death-rate of bacteria exceeds the multiplication-rate, so that the viable count decreases.

In the presence of glucose, in amounts which are relatively small, but which exceed a definite limit, bacterial multiplication is markedly accelerated in its earlier phases, but is soon retarded, and replaced by a rapidly increasing death-rate; so that the viable count decreases to a very low value, and the culture soon becomes sterile.

The rate of production of hydrogen sulphide varies in a very similar way, according to the presence or absence of a fermentable carbohydrate. In the presence of 2 per cent. glucose, for instance, the rate of production is markedly accelerated during the earlier phases of the experiment, but practically ceases after the 24th hour. In peptone media without glucose the rate of production of hydrogen sulphide is far slower during the earlier phases, but continues for a much longer period.

An obvious suggestion is that this correlation is the expression of a common underlying cause; that the glucose provides a ready source of energy for bacterial growth and activity, and that the consequent acceleration of bacterial growth and metabolism leads to increased production of hydrogen sulphide as the result of protein cleavage. We are, however, precluded from accepting this hypothesis, as affording any complete description of the course of events, by the results obtained in those experiments in which the amount of glucose in the medium was varied over a wide range. These results showed clearly that amounts of glucose, too small to influence the rate of bacterial multiplication, sufficed markedly to accelerate the rate of hydrogen sulphide production.

In plain peptone, both the hydrogen sulphide and the amino nitrogen steadily increase. This shows that both the nitrogen and the sulphur fractions of the protein complex are suffering degradation. The increase in the amino nitrogen may be due to the fact that the production is in excess of the amount utilisable, or to the fact that it is an end-product of bacterial metabolism under these conditions, in which case an increase in the amino nitrogen will indicate increased proteolysis proportionately to the amount of amino nitrogen found.

In the presence of glucose the production of hydrogen sulphide is increased, whilst the amino nitrogen remains almost stationary in amount. This may be due to increased utilisation of amino nitrogen, thus indicating increased proteolysis along this line of cleavage, or it may be that the reaction as regards nitrogen splitting is decreasing at the expense of an accelerated decomposition of the sulphur-containing fraction of the protein.

With very small amounts of glucose present, hydrogen sulphide is still formed rapidly and in large amount, whilst the amino nitrogen is stationary as determined by the Sørensen method, but if the amount of glucose be still further decreased a position is reached where a fall in hydrogen sulphide production is associated with an increase in the amino nitrogen figures. From this it seems that the glucose stimulates the attack on the sulphur fraction of the protein, and so causes a diversion of the original reaction.

Thus it seems that in a plain peptone medium both the nitrogen and sulphur fraction of the complex are attacked slowly, but on addition of glucose the sulphur complex is more vigorously attacked; so that, as measured by the degradation of the sulphur complex, the addition of glucose appears to increase proteolysis. Whether the actual rate of proteolysis is influenced by the presence of carbohydrates, cannot be determined from these results.

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ENTERITIS DUE TO *B. DYSENTERIAE* SONNE

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IN recent years outbreaks of dysentery and enteritis have been found, on occasion, to be due to late lactose fermenting organisms allied to the Flexner dysentery group. Sonne (1915) found that the main cause of dysentery in Copenhagen was a late lactose fermenting bacillus. D'Herelle (1916) in France and Øhnell (1918) in Sweden also found this atypical organism to be associated with cases of dysentery. Andrewes (1918) suggested the name *B. dispar* for lactose fermenting members of the dysentery group, which he obtained from cases of suspected dysentery and from convalescents. Thjøtta (1919) in Norway while investigating cases of dysentery obtained 40 strains of Flexner dysentery bacilli (Thjøtta group II) and 25 strains of the Sonne type (Thjøtta group III). He explained that the less frequent finding of the Sonne type was due to the fact that this organism often caused a mild diarrhoea that was not sufficiently serious to necessitate the services of a physician, with the result that the cases were not subjected to bacteriological investigation. He showed that the Sonne bacilli had the following characteristics, viz.: Large irregular crenated colonies grew on litmus lactose agar plates; acid was produced in maltose and glucose, and occasionally in lactose; no indol was produced; and serologically the group showed no relationship to the other groups of dysentery bacilli (Flexner and Shiga).

In Japan Mita (1921) isolated from children who clinically were suffering from dysentery, bacilli similar in their cultural characteristics to the type described by Sonne. These strains he called para-dysentery bacilli. In a further paper Thjøtta and Sundt (1921) showed that the Sonne bacillus produced both an endotoxin and an exotoxin. The endotoxin was the most marked in effect and produced intestinal symptoms in rabbits and mice. The exotoxin was mild in its action as compared with the exotoxin of *B. dysenteriae* Shiga and produced paresis in rabbits while mice reacted non-specifically to it. In Australia, Paterson and Williams (1922) recovered the Sonne bacillus from patients suffering from enterocolitis, dysentery, and summer diarrhoea. They found that this organism produced acid in lactose peptone water in from seven to ten days but after repeated sub-culture the acid production occurred earlier. The bacilli were agglutinated in low dilution by a monovalent serum prepared from the X strain of Flexner dysentery bacilli of Andrewes and Inman (1919) but the absorption test showed that the homologous agglutinins were not removed. More recently Bamforth (1924) has described a small outbreak of dysentery due to a late lactose fermenting type. The serological relationship of the causative organism to the Sonne bacillus was not established.

*Clinical Features of Cases.* In December 1923 a small outbreak of enteritis occurred in a ward of the City Hospital, involving within a period of 24 hours four infants whose ages ranged from 5 to 15 months.

The first case occurred on the evening of 28th December and the remaining three in the course of the next day. Attention was drawn to the condition by an elevation of temperature accompanied by a corresponding acceleration of the pulse rate. The maximum temperature was attained within 24 hours and varied from 100.4° to 101.8°. Abdominal distension and subsequent passage of mucus were common features, while two of the cases showed traces of blood in the stools. Abdominal pain was not a noticeable symptom, nor was diarrhoea a prominent feature. The symptoms lasted from 36 hours to 4½ days and recovery took place in every case, only one patient showing loss of weight as a result.

*Bacteriological Findings.* From the faeces of two out of the four cases non-lactose fermenting colonies were obtained on McConkey plates at the first examination. The colonies were not numerous, two colonies being obtained on one plate and one on another. The colonies were larger than those of the true dysentery bacilli and when the strains were replated on agar showed markedly crenated edges. Growth on an agar slope showed no special characteristics. The two strains, tested immediately after isolation, gave the following fermentation reactions:

	Allan strain			Hendry strain		
	1	8	10	1	8	10
Lactose	0	S.A.	A.	0	S.A.	A.
Mannite	A.	A.	A.	A.	A.	A.
Glucose	A.	A.	A.	A.	A.	A.
Dulcitate	0	0	0	0	0	0
Saccharose	0	0	0	0	0	0
Sorbitate	0	0	0	0	0	0
Salicin	0	0	0	0	0	0
Milk	A.	A.	A.	A.	A.	A.

A.=Acid. S.A.=Slight acid. 0=No change.

The organisms were found to be non-motile. They did not produce indol, and did not liquefy gelatin. Lead acetate medium showed definite blackening and both strains were capable of reducing nitrates to nitrites.

Two months after the Allan and Hendry strains had been isolated, *B. dysenteriae* Sonne (No. 268) was obtained from the National Collection of Type Cultures and the three strains were retested against an extended series of sugars. The strains were incubated for 14 days and the summarised results of the fermentation tests are as follows:

#### A. *Monosaccharides.*

##### 1. Hexoses.

Dextrose, laevulose, galactose, mannose.

All strains produced acid in 24 hours.

##### 2. Pentoses.

Arabinose, xylose.

All strains produced acid in 24 hours in arabinose, but no change occurred in xylose.

## 3. Methyl Pentose.

Rhamnose.

All strains produced acid in 24 hours.

B. *Disaccharides*.

## 1. Maltose.

All strains produced acid in 24 hours.

## 2. Lactose.

*B. dysenteriae* Sonne No. 268 produced slight acid on the third day and very definite acidity after five days' incubation. The Allan and Hendry strains produced a slight acid change on the second day and the medium was markedly acid on the third day.

## 3. Saccharose.

Acid was produced by all strains in 24 hours. At the first test the Allan and Hendry strains produced no acid in 10 days.

## 4. Trehalose.

All strains produced acid in 24 hours.

C. *Trisaccharides*.

## 1. Raffinose and Melezitose.

No acid production occurred with melezitose but *B. dysenteriae* Sonne No. 268 produced acid in raffinose in 24 hours. The other strains did not cause any change in 14 days.

D. *Polysaccharides*.

## 1. Dextrin.

All strains produced slight acid after 24 hours' incubation but the medium became definitely alkaline in three days.

## 2. Starch and Inulin.

No change occurred.

E. *Alcohols*.

Mannitol, Glycerol, Dulcitol, Sorbitol.

All strains produced acid in mannitol in 24 hours, and all strains produced slight acid in glycerol on the third day and definite acidity by the fifth day. The media containing dulcitol and sorbitol were unaffected by any of the strains.

F. *Glucosides*.

Salicin and Inosite.

No change occurred.

After isolation the Allan and Hendry strains were found to be pathogenic for rabbits. One-fifth of an agar slope culture when given intravenously killed rabbits in 24 hours and 2 c.c. of a killed broth culture also produced this effect. The result of intraperitoneal inoculation of guinea pigs and mice was uncertain, some of the animals surviving.

Blood was obtained from all four patients ten days after the commencement of the illness. The serum of one patient, from whom the bacillus was not

isolated, but who was ill for 4½ days agglutinated the Allan and Hendry strains to 1 in 240 and 1 in 480 respectively. The serum from the patient Hendry agglutinated the Hendry and Allan strains in a dilution of 1 in 30. Ten normal sera gave no agglutination against the Allan and Hendry strains in a dilution of 1 in 30. The sera from the patients were tested against the V, W, X, Y and Z strains of Flexner bacilli and against *B. dysenteriae* Shiga but no agglutination was obtained in a dilution of 1 in 60.

Agglutinating sera were prepared for *B. dysenteriae* Sonne No. 268 and for the Allan and Hendry strains. The serum for *B. dysenteriae* Sonne agglutinated the Allan and Hendry strains to titre (1 in 1500), the Allan serum agglutinated strains No. 268 and Hendry to titre (1 in 1000), and the Hendry serum agglutinated strains No. 268 and Allan to titre (1 in 2000).

Absorption of agglutinins showed the following:

Serum	Titre	Titre after absorption with Strain No. 268	Titre after absorption with Strain Allan	Titre after absorption with Strain Hendry
No. 268	1500	nil.	nil.	nil.
Allan	1000	nil.	nil.	nil.
Hendry	2000	nil.	nil.	nil.

The strains were tested against monovalent sera prepared from *B. dysenteriae* Flexner V, W, X, Y, and Z, and *B. dysenteriae* Shiga. The V serum which had a titre of 1 in 20,000 agglutinated all the strains (No. 268, Allan and Hendry) to a titre of 1 in 400 but the absorption agglutinin tests showed that the homologous agglutinins were not removed. Agglutinating sera prepared against *B. typhosus*, *B. paratyphosus* A, *B. paratyphosus* B, and *B. enteritidis* Gaertner had no action on any of the strains.

The effort to trace the source of infection was unsuccessful, the faeces of the other patients in the ward and of the nursing staff in charge of the children being examined with negative results.

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# THE RESPECTIVE INFLUENCES OF TEMPERATURE AND MOISTURE UPON THE SURVIVAL OF THE RAT FLEA (*XENOPSYLLA CHEOPIS*) AWAY FROM ITS HOST<sup>1</sup>.

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(With 3 Text-figures.)

THE rapid fall in the number of plague cases with the onset of hot weather is a characteristic feature of plague epidemics in the northern half of India. In these regions the rise in temperature is accompanied by an increased drying capacity of the atmosphere so that it is impossible to assess to what extent the higher temperature and increased drying power are respectively responsible for the effect.

Epidemiological observations made in Mauritius (1899), Southern India (1908) and Java (1914) suggest that a rise of temperature, without increased drying power of the air, restrains plague epidemics. These observations have been ably analysed by Brooks (1917). The object of the experiments set forth below was to ascertain the separate influences of temperature and drying upon the longevity of fleas with a view to the interpretation of the epidemiological facts.

To save possibility of confusion we may be permitted to point out that the meteorological term relative humidity does not indicate the drying capacity of an atmosphere. Drying power depends on the saturation deficiency, that is the extent to which the vapour pressure of water in the air is short of the saturation pressure for the particular temperature. Investigation both by members of the Plague Commission in India (1912) and by Bacot (1914) in this country have shown that considerable saturation deficiency exerts an inimical influence at almost every stage of the life history of the insect and the consequent diminution in flea-population no doubt plays a considerable part in modifying the spread of plague. Our observations are only concerned with the effect upon the longevity of the adult insect apart from its host.

Whilst living amongst the fur of an animal the insect is exposed to a nearly constant climate. Also, it can slake its thirst whenever it is so disposed. Once separated from its host, however, the time which elapses before its career is ended by desiccation is principally determined by the drying power of the atmosphere. This is abundantly illustrated by the experiments in the reports referred to above and also by the observations of Nicoll (1912) and of Petrie (1923) in Egypt.

Apart from the general reduction in flea population, saturation deficiency

<sup>1</sup> The experiments recorded in this paper were made in 1914. The work was put aside during the war. For various reasons, amongst them the death of Arthur Bacot, who contracted typhus whilst experimenting with infected lice, the observations have not been recorded until now.

has a special significance in the epidemiology of plague, for when a rat dies of plague its fleas have, perforce, to roam in search of another host and the longer the time vouchsafed to them, the greater the chances of a successful quest.

The time of survival is abbreviated in the case of fleas in which the lower end of the gullet is obstructed by cultures of plague bacilli. Such fleas are particularly sensitive to climatic conditions as they may be already a bit dehydrated. Although they bite continuously they find great difficulty in satisfying their thirst. The action of the pharyngeal pump distends the oesophagus with blood but little or none enters the stomach and on the pump ceasing to function the blood flows back into the wound carrying with it plague bacilli from an infected gullet. We have shown elsewhere (1914) that insects in this condition are principally responsible for the transmission of infection from rat to rat and presumably from rat to man. We could, however, keep fleas alive for weeks when the entrance to the stomach was completely blocked if the atmosphere were nearly saturated and the temperature below 5°.

The Poona observers attempted to answer the question whether temperature, apart from humidity, exerted any direct influence upon the length of life of fleas, by reducing the latter to nothing. They kept fleas at different temperatures in test-tubes in which the air was dried by calcium chloride and found that at 101° F. the mean duration of life was 0.14 day whereas at room temperature (55–85° F.) it was 1.17 days and in a cool box at 60° F. it was 1.66 days. The influence of temperature could hardly be arrived at in such a manner, for, assuming the air in the tubes to have been dry, the saturation deficiency was different at each temperature. It would be 50 mm. at 101° F., 18.7 mm. at 70° F., and 13.6 mm. at 60° F. In addition to changing the temperature of the fleas the observers had unintentionally varied the drying power of the atmosphere to the maximum extent.

What we set out to do was (1) to keep the saturation deficiency constant while temperature was varied, (2) to keep temperature constant while saturation deficiency was varied and so determine the effect of each of these variables upon the longevity of the insects.

#### METHODS EMPLOYED.

The most convenient way of arranging atmospheres having the same temperature but different saturation deficiencies or the same saturation deficiency but different temperatures is to bring them into equilibrium with various mixtures of sulphuric acid and water. If, for instance, air is slowly bubbled through water at 10° C., it takes up water to the maximum vapour pressure for that temperature, viz. 9.2 mm. If it is bubbled through pure  $\text{H}_2\text{SO}_4$  its vapour pressure is reduced to zero. By arranging the proportion of acid to water in the mixture through which the air is passed, any intermediate vapour pressure can be arrived at.

From Regnault's data which are given in Landolt and Börnstein's tables (1905 edition, p. 166) the mixture required to give a particular vapour pressure at a particular temperature can be calculated. This is the procedure we adopted. The fleas were in a bottle which was maintained at a constant temperature through which a constant stream of air of the same temperature and the desired saturation deficiency was drawn.

One hundred fleas from the breeding cage were placed in small test tubes about an inch long, ten fleas to each tube. The tubes were covered with gauze and suspended in a cage inside a wide-necked bottle of about 700 c.c. capacity. At the bottom of the bottle was 200 c.c. of a mixture of sulphuric acid and water in the required proportion. The neck of the bottle was closed with a rubber bung penetrated by two glass tubes, one of which dipped beneath the acid. Air was drawn through the bottle, bubbling through the sulphuric acid mixture. As merely bubbling through a couple of inches would not bring the vapour pressure in the air into equilibrium with that of the mixture of sulphuric acid and water, the air was passed through two other gas-washing bottles containing the same strength of sulphuric acid at the same temperature before entering the bottle containing the fleas. This, we ascertained, sufficed to obviate change in the concentration of acid in the final bottle. A constant temperature was secured by immersing the whole apparatus in a large water bath controlled in the usual way. The bottle was examined twice daily. The dead fleas were counted and removed and the bottle replaced in the bath.

The fleas used in the experiments were *Xenopsylla cheopis* bred in the laboratory. They were taken from the breeding cages immediately before use and represented a mixed population of various ages and in different states of nutrition.

We employed throughout a flow of 100 c.c. per minute. The details of the procedure for controlling saturation deficiency and temperature will be grasped from an example. Supposing it were desired to subject the fleas to a current of air of 100 c.c. per minute at the temperature  $32^{\circ}\text{C}$ . and with a saturation deficiency of 10 mm. mercury pressure, *i.e.* a vapour pressure of 25.6 mm.

The saturation vapour pressure at  $32^{\circ}\text{C}$ . is 35.6 mm.  $35.6 - 10 = 25.6$  mm. 32 per cent.  $\text{H}_2\text{SO}_4$  solution at  $32^{\circ}\text{C}$ . has a vapour pressure of 25.6 and air brought into equilibrium with acid of this strength will have this vapour pressure and, *as long as it be maintained at  $32^{\circ}\text{C}$ .*, a saturation deficiency of 10 mm.

Air was passed firstly, through a gas-washing bottle containing water, immersed in a bath at  $27^{\circ}\text{C}$ . The vapour pressure at  $27^{\circ}\text{C}$ . is 25.6 mm. and the issuing air had a vapour pressure of about 25 mm. For the final adjustment of its vapour pressure, it was driven through two gas-washing bottles containing 32 per cent.  $\text{H}_2\text{SO}_4$  and thence into the bottle containing the fleas, bubbling through more 32 per cent.  $\text{H}_2\text{SO}_4$  in the bottom of this bottle. These last two gas-washing bottles and the bottle containing the fleas were immersed in a bath at  $32^{\circ}\text{C}$ . The various bottles were connected by short lengths of

rubber tube. To secure a constant stream of 100 c.c. per minute, the air was delivered at constant pressure by means of an overflow valve through a gas meter. The flow was adjusted by regulating the resistance.

#### PRELIMINARY TEST OF APPARATUS.

Within the range of temperature we are considering, air with the same saturation deficiency and the same movement dries an object at very nearly the same rate, whatever the temperature. There is a small advantage at the higher temperature because molecules diffuse slightly more quickly. When, as in our experiments, the difference in temperature is only  $11^{\circ}\text{C}$ . the increase is less than 2 per cent.

To test our apparatus and the correctness of our mixtures of sulphuric acid and water we determined the rate of drying of small discs of asbestos paper suspended in the bottle destined for the flea experiments. A disc was placed in one of each of three of the small test tubes and two drops (about .04 c.c.) of water were allowed to drop on to each from a capillary pipette. The tubes were placed in stoppered weighing bottles, weighed and at once put in the cages suspended in the wide-necked bottle which was immersed in the bath at  $32^{\circ}\text{C}$ . and a current of air with a saturation deficiency of 10 mm. driven through the apparatus at the rate of 100 c.c. per minute. After 10 hours the tubes were removed, placed in their corresponding weighing bottles and again weighed. The results were as follows:

	Weight before	Weight after	Loss by evaporation
1	19.7214	19.7066	.0148
2	26.4704	26.4554	.0150
3	20.6190	20.6044	.0146
		Mean	.0148

The same experiment was repeated in a bath at  $21^{\circ}\text{C}$ . with the same air current and the same saturation deficiency obtained in this case by bubbling through 45.57 per cent.  $\text{H}_2\text{SO}_4$ .

	Weight before	Weight after	Loss by evaporation
1	17.4035	17.3889	.0146
2	17.6377	17.6231	.0146
3	16.4447	16.4303	.0144
		Mean	.0145

A 2 per cent. difference in favour of the higher temperature but hardly outside the experimental error.

#### THE LOSS OF WEIGHT OF FLEAS ON DESICCATION.

As fleas are covered with chitin one might conjecture that they would be resistant to drying. Such, however, is far from the case. As will be seen from the following table, fleas from the breeding cage lost half their weight by



*The Survival of the Rat Flea*

evaporation in four days at 32° C. (90° F.) in an atmosphere the saturation deficiency of which was only 10 mm., i.e. a relative humidity of 72 per cent. at this temperature. The movement of the air through the bottle was so slow that it may be regarded as nil. By this time 84 per cent. were dead and the remainder died before the loss of water reached 60 per cent. of their own weight. The initial water content of the insects was found to be 80 per cent.

*Rate of Loss of Water of 50 Fleas.*

T. 32° C. Saturation deficiency 10 mm.

Time (hrs.)	Weight 50 fleas	Loss of H <sub>2</sub> O	% loss of weight	Survivors
0	·0308	—	—	50
2	·0288	·0020	6·5	50
4·5	·0284	·0024	7·8	50
20	·0250	·0058	18·8	48
25	·0240	·0068	22·1	43
46	·0206	·0102	33·1	34
51	·0198	·0110	35·7	33
67	·0180	·0128	41·5	20
100	·0148	·0160	51·9	8
125	·0136	·0172	56·0	4
142	·0130	·0178	57·8	0
Dried in desiccator to constant weight	·0062	·0246	80·0	—

The graphs (Fig. 1) in which the results in terms of the percentage loss of weight of fleas and their survival are plotted as ordinates against time in hours show the relation of mortality to drying.

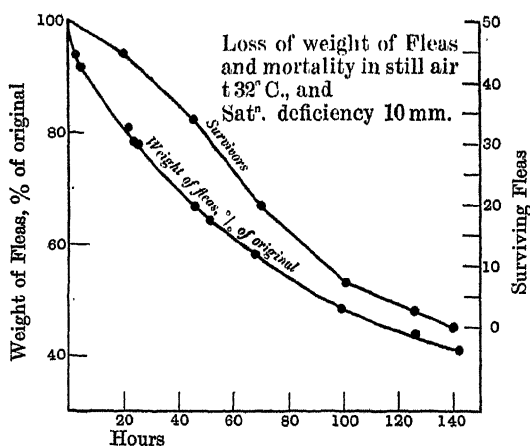


Fig. 1.

THE INFLUENCE OF VARYING SATURATION DEFICIENCY ON THE LONGEVITY OF FLEAS, TEMPERATURE BEING CONSTANT.

A mixed population of fleas, *X. cheopsis*, from the cages were used, 100 for each experiment. In all the experiments the temperature was 32° C. (89·6° F.) and the air current through the bottle 100 c.c. per minute.

The observations were made with saturation deficiencies of 4, 10, 16 and

26 mm. produced by bubbling the air through appropriate mixtures of sulphuric acid and water. At 32° C. these saturation deficiencies correspond to relative humidities of 89, 72, 55 and 27 per cent., *i.e.* from highly saturated to what would be very dry air for natural conditions.

The results are expressed in the series of graphs in Fig. 2 in which the number of surviving fleas is plotted against time in hours. Each graph has the same general characteristic, the descent being steeper as the saturation deficiency increases. The area included between each of the curves and the ordinate and abscissa is proportional to the mean life of the fleas at the particular saturation deficiency.

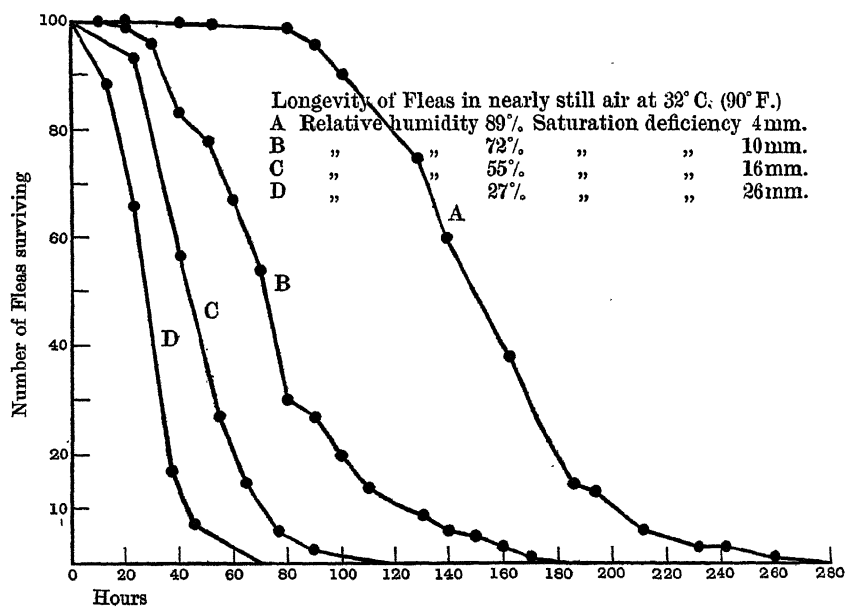


Fig. 2.

The relation of mean life to saturation deficiency is shown in the following table the figures in which are derived by computing these areas after smoothing the curves:

Saturation deficiency in mm.	Mean life in hours	Mean life x Saturation deficiency
4	152	608
10	68	680
16	44	704
26	27	702

By multiplying the saturation deficiency by the mean life a nearly constant number is obtained in the case of the higher saturation deficiencies. This product, however, diminishes considerably at 4 mm. as saturation is approached. Indeed, a departure must occur, for the fleas do not live for ever in saturated air when the saturation deficiency is zero.

We conclude, however, that, apart from this limitation, the mean life of

the insects varies inversely as the saturation deficiency. As the rate of drying also varies inversely as the saturation deficiency, this signifies that, at constant temperature, the mean life of fleas apart from their host is inversely proportional to the rate at which they lose water by evaporation.

THE INFLUENCE OF TEMPERATURE ON THE LONGEVITY OF FLEAS WHEN  
THE SATURATION DEFICIENCY IS KEPT CONSTANT.

We made but two experiments to determine this, one at 32° C. (90° F.), the other at 21° C. (70° F.). In general arrangement the method was the same as has been described above but the temperature of the bath in the one case was maintained at 21° and in the other at 32° C. In the former the preliminary moistening of the air was omitted, as bubbling it through three bottles containing 45·5 per cent.  $\text{H}_2\text{SO}_4$  in succession was adequate to secure and maintain a vapour pressure of 8·5 mm. in the bottle containing the fleas. This vapour pressure at 21° C. leaves a saturation deficiency of 10 mm. As before, 100 fleas, were used for each experiment. The results are shown in the graphs in Fig. 3

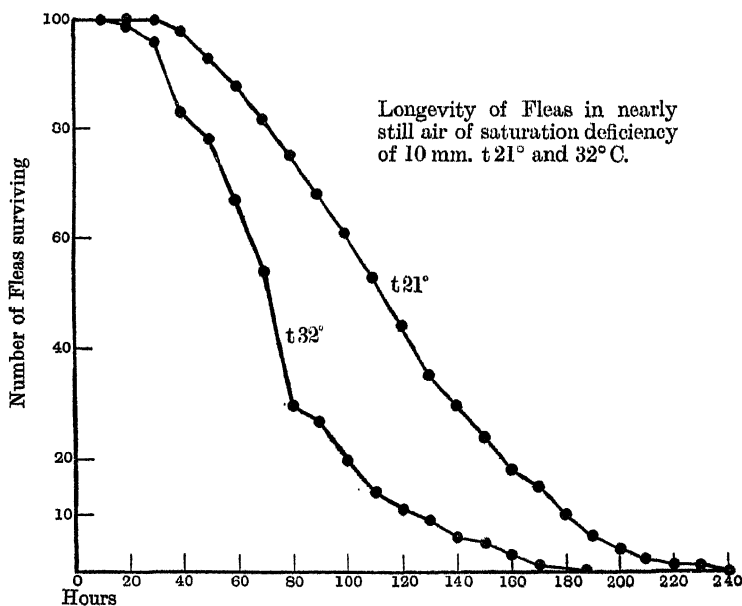


Fig. 3.

which represent the number of fleas alive after various intervals. The mean life of the insects at the two temperatures, derived, as mentioned above, after smoothing these graphs, is 115 hours at 21° C. and 68 hours at 32° C., that is, the fleas lived 1·78 times as long at the lower temperature although they were presumably losing water at the same rate, as the saturation deficiency was the same. This diminished length of insect life at the higher temperature is probably an instance of a general biological law. Those biological activities which have been studied quantitatively in insects are about doubled

by a rise of  $10^{\circ}\text{C}.$ <sup>1</sup> and it has also been shown that a rapid life entails a short one. Loeb and Northrop (1917) found that the life of the imago of the fruit-fly (*Drosophila*) at  $30^{\circ}\text{C}.$  was one-third of that at  $20^{\circ}\text{C}.$

#### CONCLUSIONS.

1. The survival of fleas (*X. cheopis*) apart from their host is approximately in inverse proportion to the saturation deficiency of the air, provided the temperature and air movement are constant. In other words, it is proportional to the rate at which they lose water.

2. Under similar conditions but with constant saturation deficiency, their length of life is reduced to between one-half and two-thirds by  $10^{\circ}\text{C}.$  rise in temperature. Compared with the effect of saturation deficiency, that of temperature upon the longevity of fleas is, within the range of climatic conditions over the greater part of India, a smaller one.

3. A variation in saturation deficiency from 5 mm. to 35 mm. such as occurs in the plains of Northern India at different seasons would, accordingly, shorten the average duration of life of wandering rat fleas in the proportion of 15 to 1. As a rise in mean temperature occurs simultaneously with the increase in saturation deficiency and may amount to a difference of  $20^{\circ}\text{C}.$  between January and June this would reduce the length of life of wandering fleas to about one-third. The effect of saturation deficiency and increased temperature will be additive and would go a long way to explain some of the climatological features of the epidemics.

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<sup>1</sup> A useful collection of data is in Kanitz, *Temperatur und Lebensvorgänge*, Berlin, 1915.

## THE BACTERIOLOGICAL EXAMINATIONS OF THE FAECES IN FOUR CASES OF TYPHOID FEVER, MADE AT FREQUENT INTERVALS FOR A PERIOD OF ONE MONTH.

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### REASONS FOR INVESTIGATION.

THE investigations described in this paper were designed in order to observe whether changes in the diet of patients suffering from typhoid fever were accompanied by any changes in the faecal flora, and also to observe whether pyrexia, diarrhoea or constipation occurring during the course of the disease altered the numbers of typhoid bacilli in the faeces.

The investigations were confined to four cases of the disease, which were under the care of Dr Foord Caiger at the South Western Fever Hospital. This fact precluded any chance of there being any articles of diet administered other than those prescribed.

The observations were controlled by examinations of the urine and the specimens of faeces were very carefully taken so as to avoid urinary contamination.

### CLASSIFICATION OF THE INTESTINAL FLORA.

The intestinal flora has been classified in many ways by various investigators. Cushing and Livingood (1900) divided it into two groups, (1) permanent or obligatory, and (2) transient or facultative. The first group being always present, the second unable to flourish in the presence of the first, unless it encounters or produces a pathological lesion of the mucosa. The conditions set up by infection with the *B. typhosus* are, these workers maintain, of such a nature as to encourage additional members of this second group to flourish.

Torrey (1919) divided intestinal flora into two groups, (1) fermentative, and (2) putrefactive; with *B. coli* mid-way between and having the power of passing from one to the other main group. He classed *B. Welchii*, *B. acidophilus*, *Streptococcus* (including *Enterococcus*) and some others in the first group, while he placed *B. sporogenes*, *B. proteus*, *B. pyocyaneus* and *Staphylococci* in the second.

Morris, Porter and Meyer (1919), in an elaborate and comprehensive investigation, divided the intestinal flora into three groups, (1) fermentative or

saccharolytic, (2) putrefactive or proteolytic, and (3) facultative or normal. In the main these workers supported the views of Torrey (1919), but they sharply differentiated the faecal flora of young children from that of adults, the former, according to them, being chiefly of group 1, the latter tending, as age advances, to become more and more of the type of group 2. They maintained that this fact bears greatly on the course and prognosis in cases of typhoid fever.

#### THE BACILLUS TYPHOSUS.

Typhoid bacilli make their appearance in the intestinal canal as a result of the ingestion of infected food or water. Penetrating the mucosa they invade the blood stream and an extensive infection of the intestinal lymphoid tissue takes place.

#### OTHER ORGANISMS.

The commonest inhabitant of the intestine, both normally and in cases of typhoid fever, is the *B. coli*.

Lembke is quoted by Dudgeon (1924) as having found that the *B. coli* alone remained constant and independent of diet in 89 cases examined. This, however, was before various strains of *B. coli* were recognised and before it was realised that this organism is capable of assuming either a fermentative or proteolytic character, lying, according to Torrey (1919), mid-way between these two great groups of intestinal bacteria.

Dudgeon, Wordley and Bawtree (1921 and 1922) have further classified the *B. coli* into two other groups, namely haemolytic and non-haemolytic. The non-haemolytic colon bacilli occur in the intestinal tracts of 85 to 90 per cent. of normal persons, whereas in such conditions as colitis and diarrhoea the haemolytic variety may be found in as many as 35 per cent. of the specimens of faeces examined.

Streptococci are very common normal inhabitants of the intestinal tract, and in this series of four cases of typhoid fever an abundance of these organisms was found, which however diminished rapidly as the amount of milk in the diet was decreased. The cause of this is, in all probability, as is pointed out by both Torrey (1919) and by Dudgeon (1924), directly due to the rich supply of streptococci in the milk.

Kufferath (1921) in an investigation extending over four years found streptococci in 21 per cent. of the specimens of dairy milk submitted to him. Frost and Bachmann (1922) found haemolytic streptococci in 28 per cent. of specimens of high grade milk obtained from 412 cows.

Many other organisms may be isolated from the stools of typhoid patients, the commonest being *Staphylococcus albus* and *aureus*. According to Torrey (1919), these belong to the proteolytic type and would be expected more in putrefactive than in fermentative stools.

## THE SIGNIFICANCE OF SECONDARY ORGANISMS.

It is necessary to determine as far as possible what conditions cause changes in the flora of the intestine, and what effect such changes have on the patient, especially in cases of typhoid fever.

A quotation from Herter (1907) in this connection may not be out of place. "Our attention has, perhaps, been too exclusively fixed on the specific excitants and the rôle played by associated bacteria must receive more study, for it is clear that they sometimes play a significant part in determining the outcome of an infection. The difference that decides whether a man shall live or die must frequently be a slight one looked at from the standpoint of the processes of battle within the body."

It is well known that there are many septic conditions which occur as complications of typhoid fever from which it is possible to cultivate the *B. typhosus*. A great majority of the abscesses and boils, however, are caused by staphylococci. It is interesting to note that in two of the cases which I investigated boils developed during the attack of typhoid fever. The stools from case number 2 had previously contained a large number of *Staphylococcus aureus* with an entire absence of *S. albus*; the pus from the boils in this case showed a pure culture of *S. aureus*. The faeces from case number 4 showed an abundant growth of *S. albus*, and the boils in this case contained this organism also in pure culture.

Prof. L. S. Dudgeon has shown me records of some of his cases of typhoid fever in which suppurative conditions occurred in various parts of the body due to *S. aureus*, which organism was in each case isolated in abundance from the faeces. One case in point was diagnosed from blood-culture as typhoid fever, but no typhoid bacilli, haemolytic *B. coli* or *S. aureus* were found in the stools; fifteen days later a large abscess developed in connection with an acute osteo-myelitis of the right femur; from this abscess *S. aureus* was grown in pure culture while the stools contained this organism in abundance.

## THE RELATION OF DIET TO FLORA.

The types of intestinal flora are chiefly controlled by the chemical character of the food. Carbohydrates favour on the whole fermentative organisms, protein, on the other hand, favours putrefactive ones.

The most powerful of the carbohydrates in suppressing the proteolytic bacteria are lactose and dextrin. A diet of bread and milk, which contains these two carbohydrates, sets up a flora of the fermentative type in a very short space of time, provided always, as is shown by Torrey, that the calorie content is high enough. Torrey (1915) with great care and detail examined 100 stools from 22 typhoid patients. He shows that with a diet rich in carbohydrates (carbohydrate 250-300 gms., protein 50-100 gms., fat 75-100 gms. per diem) a fermentative state can be induced in the canal, which, by the acid reaction that it induces, is inhibitory to the growth of the proteolytic

bacteria and thus aids in preventing absorption of toxic bodies to which these organisms give rise. He shows that the ordinary milk diet, with its low calorie content, is not able to support a fermentative state, let alone induce one, in a subject having a tendency to a proteolytic flora. He points out that although the state of the flora does not influence the susceptibility to attack, yet, in those patients who, on admission to hospital, had a flora of the fermentative type, the disease tended to run a milder course. He considers this to be the reason why young children often have milder attacks of typhoid fever than adults. It would seem from this investigation by Torrey that diet has a very marked influence on the intestinal flora, and bearing in mind the remarks of Herter (1907) quoted above, it is reasonable to think that in the past the relation between diet and intestinal flora has not received due attention.

It was the observations of Torrey (1915) and of Dudgeon (1924) that led to the investigation of this series of four cases of typhoid fever in an endeavour to ascertain whether there are any modifications indicated in the diet usually given to typhoid patients in this country.

#### CASES.

The four cases which I investigated occurred in members of one family. The first individual to be affected was a child, and there followed a fortnight later an infection in the mother and in two other children. The father and the two remaining children were not attacked. The cause of the outbreak was believed to be certain infected clothing in the marine-store kept by the family. I am indebted to the kindness of Dr Foord Caiger for permission to carry out my investigations on these patients under his care.

#### SUMMARY OF THE CLINICAL HISTORIES OF THE PATIENTS.

*Case 1.* S. U., aet. 13, female, admitted to hospital 20. xii. 23 with 14 days' history of illness diagnosed clinically as typhoid fever.

Patient looked ill; abdomen distended but not tender; spleen not palpable. Temp. 102.5, pulse 130.

Patient remained acutely ill for 12 days. Serum taken on the 22nd day agglutinated *B. typhosus* 1/100, but not *B. paratyphosus* A, B and C. Temp. 28th day, normal. Uninterrupted recovery.

*Case 2.* E. U., aet. 41, mother of the other patients, admitted 5. i. 24 with 10 days' history of illness; abdomen distended but general condition good. Temp. 101.2, pulse 109.

Serum on 18th day agglutinated *B. typhosus* 1/400, but not *B. paratyphosus* A, B and C. General course of the disease mild; temp. 20th day, normal.

*Case 3.* C. U., aet. 11, daughter of above, admitted 5. i. 24 with 7 days' history of illness. Patient looked ill but had no distension. Temp. 103, pulse 100.



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Spleen palpable 12th day; rash appeared 15th day; serum taken on 16th day agglutinated *B. typhosus* 1/500. General course mild; temp. 18th day, normal.

Case 4. K. U., aet. 9, sister of above, admitted 5. i. 24 with 8 days' history of illness. Patient looked ill, but had no distension. Temp. 103·5, pulse 109.

Serum taken on 15th day agglutinated *B. typhosus* 1/100, but not *B. paratyphosus* A, B and C. The general course was more severe than the preceding cases; recovery however was uninterrupted. Temp. 28th day, normal.

### TREATMENT.

All four cases were treated on the same lines. The diet consisted of: milk, 2 oz.; lime water, 2 oz.; glycerine of pepsin,  $\frac{1}{2}$  dr. and cinnamon oil, 5 m. every two hours, during the febrile period. Distension was treated with Ol. Terebinth, 5 m. every four hours. After the temperature had subsided liquid paraffin was given in each case.

### INVESTIGATION OF CASES.

The results of the investigations which I carried out are tabulated below.

#### Case 1. 18 January, 1924.

Day of dis.	<i>B. typhosus</i>	Strep. and staph.	Diet etc.	Purgatives	Stools	Temp.	Urine
43	0	+	Semi-solid with milk	Paraffin liquid	Normal	Normal	<i>B. coli anaerogenes</i>
44	0	+	"	"	"	"	+
46	0	+	"	"	"	"	+
50	0	+	Chicken, milk less	"	"	"	
54	0	+	"	"	Slight diarrhoea	"	
56	0	+	Milk less	"	Normal	"	<i>B. coli anaerogenes</i>
62	0	+	"	"	"	"	+
64	0	<i>S. aur.</i> +	"	"	"	"	
67	+	<i>S. aur.</i> + +	"	"	Slight diarrhoea	"	
69	0	+	"	"	Normal	"	
75	0	+	"	"	Slight diarrhoea	"	<i>B. coli anaerogenes</i>

#### Case 2. 18 January, 1924.

Day of dis.	<i>B. typhosus</i>	Strep. and staph.	Diet etc.	Purgatives	Stools	Temp.	Urine
23	+	+	Milk	Nil	Constipated	100 99·8	<i>B. typhosus</i> +
28	+	+	"	"	"	Normal	
30	+	+	"	"	"	"	
35	+	+	Semi-solid with milk	Paraffin liquid	"	"	<i>B. coli</i> + +
37	+	+	"	"	"	"	

## Case 2. 18 January, 1924 (continued).

Day of dis.	<i>B. typhosus</i>	Strep. and staph.	Diet etc.	Purgatives	Stools	Temp.	Urine
42	+ -	+	Semi-solid with milk	Paraffin liquid	Constipated	Normal	
44	+	+ -	"	"	"	"	
47	0	+ -	Solids, milk less	"	"	"	
49	0	+ -	"	"	"	"	
51	0	Nil	"	"	"	"	
54	0	"	"	"	"	"	<i>B. coli</i> + -

Remarks:—1. Enema simplex given daily.  
 2. 35th day *S. aureus* isolated from boils (right leg and back).  
 3. 47th day some haemolytic *B. coli* in the stools.

## Case 3. 18 January, 1924.

Day of dis.	<i>B. typhosus</i>	Strep. and staph.	Diet etc.	Purgatives	Stools	Temp.	Urine
20	0	+++	Milk	Nil	Diarrhoea	Normal	<i>B. coli</i> + +
21	0	+++	"	"	"	"	
23	0	+++	"	"	"	"	
		<i>S. alb.</i> + +					
25	0	+++	"	"	"	"	
		<i>S. alb.</i> + +					
27	+	+++	"	"	"	"	
		<i>S. alb.</i> +					
31	0	+++	"	"	"	"	
		<i>S. alb.</i> + -					
32	+	++	Semi-solid	"	"	"	<i>B. coli</i> + +
37	++	+	"	"	Slight diarrhoea	"	
44	++	+	Solids, milk less	Paraffin liquid	Normal	"	
46	0	++	"	"	"	"	
48	0	+	"	"	"	"	
51	0	+ -	"	"	"	"	<i>B. coli</i> +

## Case 4. 18 January, 1924.

Day of dis.	<i>B. typhosus</i>	Strep. and staph.	Diet etc.	Purgatives	Stools	Temp.	Urine
21	0	+++	Milk	Nil	Diarrhoea	101.5 101	<i>B. coli</i> + +
22	0	+++	"	"	"	101.5 101	
		<i>S. alb.</i> +				101	
24	0	+++	"	"	"	101 100	
26	0	+++	"	"	Normal	101 99.8	
		<i>S. alb.</i> + +					
31	++	+++	"	"	Constipated	Normal	
33	++	+	"	"	"	"	<i>B. coli</i> + -
		<i>S. alb.</i> + + +					
36	++	++	"	"	"	"	
		<i>S. alb.</i> + +					
38	++	+	Semi-solid with milk	"	"	"	
		<i>S. alb.</i> + + +					
40	0	+ -	"	"	"	"	
45	0	+++	"	Paraffin liquid	Normal	"	
47	0	+ -	"	"	"	"	
49	0	+ -	Solids, milk less	"	"	"	<i>B. coli</i> +

Remarks:—1. 36th day *S. albus* isolated from boils (shoulder and back).

In the above tables: + + + indicates "in abundance."

+ + " "many."

+ " "few."

+ - " "1 to 3 colonies only."

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### METHODS EMPLOYED.

The examination of the specimens from these four patients was carried out on the following lines.

The specimens passed in the morning were examined about two to three hours later. The general character of the stool, and its reaction to litmus was noted, a small portion of the stool was then dried by the method devised by Dudgeon and described by Wordley (1921).

This method consists of taking a specimen of faeces and transferring a portion thereof to the surface of a dry, sterile porous tile on which it is evenly spread with a knife. The tile is then placed under a bell-jar and left to dry at room temperature for two or three hours after which the material is scraped off and transferred to another similar tile and left until it is quite dry. When dry the material is scraped as a powder into a small heap in the centre of the tile, thoroughly mixed, and a little is placed on the various media upon which it is to be cultivated.

In order to increase the percentage of successful attempts to isolate the *B. typhosus*, various methods have, from time to time, been devised, such as the "Brilliant Green" method of Browning. Lactose bile has also been tried as a selective medium, but according to Tonney, Caldwell and Griffin (1916) it is directly antagonistic to the growth of the *B. typhosus*.

The tile method has a great advantage over these other methods in that it enables the maximum number of typhoid bacilli to be obtained without retarding the growth of the remainder of the flora. It is thus possible to study the complete flora with one method, and if desired it is possible to use blood-agar as a plating medium for the growth of the streptococcus and other haemolytic organisms. Another advantage is that, since the material placed on the media is a dry powder, comparisons from day to day have a common and stable basis, no matter whether the patient is constipated or diarrhoeic.

### ISOLATION AND CULTIVATION OF *B. TYPHOSUS* AND OTHER FLORA FROM TYPHOID FEVER PATIENTS.

The cultivation of the *B. typhosus* from the faeces, except in the later stages of the disease, may not be of importance in the actual diagnosis of typhoid fever, owing to the earlier and more certain methods of blood-culture and agglutination tests. It is of importance, however, in the stage of convalescence and in the case of a suspect carrier.

The media employed in this investigation were:

1. Two plates of litmus lactose agar. ("Lemco," 1 per cent., NaCl, 0.25 per cent.; peptone, 1 per cent.; agar, 2.5 per cent. and litmus 10 per cent.)
2. One plate of blood-agar (10 per cent. whole human blood).
3. One plate of agar.
4. One tube of litmus milk.
5. One tube of starch medium (1 per cent. starch in "Lemco" broth).

To each of the above a small quantity of the powdered faeces was added and all were incubated at 37° for 24 hours.

McConkey's medium was not used with the tile method because it was not desired to retard the growth of any organism, but rather to encourage every organism present in the faeces to grow on the medium so that a complete picture of the flora might be obtained. Litmus lactose agar was used therefore as a plating medium.

After incubation any blue translucent colonies on the litmus lactose plates were transferred into tubes of lactose broth and on to agar slopes. Any other colonies required for further examination, such as haemolytic colonies from the blood-agar plates, were transferred to agar slopes.

The milk tubes were examined for clot and the presence of foaming, and the starch tubes were tested microscopically for yeasts on the 1st and 3rd days.

Any gram-negative, motile organism, which after 24 hours' incubation had not produced gas in lactose, was tested by the coarse agglutination method against anti-typhoid serum and further inoculated into tubes of "Lemco" broth containing 1 per cent. of glucose, mannite, dulcitol, cane-sugar, and lactose; and also into tubes of litmus milk and peptone water.

Organisms which produced gas in lactose were tested for haemolysis against a reagent composed of human red cells suspended in peptone water containing 0.5 per cent. and 0.85 per cent. NaCl. They were also tested by the coarse agglutination method against the anti-coli sera prepared by Dudgeon, Wordley and Bawtree (1922). Three of these were of a non-haemolytic, and two of a haemolytic strain.

All the sugar reactions were read on the 1st and 7th days and the litmus lactose plates were examined at the end of 24 and 48 hours.

An approximate differential count was made of the colonies on each plate.

#### CONDITIONS AFFECTING THE ISOLATION OF TYPHOID BACILLI.

The conditions bearing on the isolation of the bacilli from the stools are (1) the stage of the disease, (2) the state of the stools at the time of the investigation, and (3) the methods employed.

Attempts to isolate the bacilli from the stools are successful, according to an average of the figures given by various writers, in about 50 per cent. of cases by the end of the third week of the disease. The state of the stools is important as regards (1) whether they are fluid or formed, (2) whether they are acid or alkaline, (3) the time that has elapsed since they were passed, and (4) the question of urinary or other contamination.

There is no doubt that the last two are the most important; the time interval between collection and examination should be as short as possible and never exceed 12 hours, and great care must be taken to avoid contaminating the faeces with urine in order that urinary organisms may not be ascribed

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to the faeces. As regards whether the stool should be fluid or not to get the best results, Tonney, Caldwell and Griffin (1916) maintain that elaterin when administered to a patient acts as a powerful cathartic without being in any way an antiseptic, and they found a much higher percentage of positive results when using this drug than they did without it.

### RESULTS OF THE INVESTIGATION.

The appearance of the *B. typhosus* in the faeces did not show any relationship to changes in the diet or treatment in any of the four cases.

The later in the disease that the bacilli appeared, the shorter was the duration of their appearance.

Accompanying the appearance of the bacilli in Case 1 on the 67th day there was an abundant growth of slow lactose fermenting *B. coli*, forming very large colonies.

The disappearance of the *B. typhosus* from the stools was followed in each case by a marked simplification of the intestinal flora.

Typhoid bacilli were isolated in all 15 times from these four cases. Case 2, who was constipated during the whole investigation, gave seven consecutive positive results, and I think that this fact indicates that constipation *per se* is not detrimental to success when the tile method is used.

The mildest case clinically (Case 2) had most typhoid bacilli, both faecal and urinary.

Streptococci diminished in number in all these cases *pari passu* as the milk in the diet was reduced.

Haemolytic streptococci and staphylococci were found on nine occasions. Yeasts were never isolated, but sarcinae were frequently found.

The finding of the same organism in the bowel and in the skin lesions has already been referred to.

A few special points may be worthy of notice such as the frequent presence of *B. coli anaerogenes* in the stools and urine of Case 1. Another interesting point was the occurrence in all four cases, at or about the same time, of an abundant growth of the slow lactose fermenting *B. coli* referred to above; of these the organisms isolated from Case 2 were strongly haemolytic and were agglutinated by one of the haemolytic anti-*coli* sera in the series referred to above.

The bacilluria of typhoid bacilli in Case 2 when treated with hexamine gave place to one of *B. coli*.

The daily changes in flora in all the cases were slight and the general type was simple. *B. coli* was the only organism which was invariably present, though at times more abundant than at others. *S. aureus* was present on only six occasions. The stools throughout were slightly alkaline to litmus. Had these patients been given the diet advocated by Torrey (1919) what changes might we have expected, either clinical or bacteriological? I fail to see that

any material change would have resulted, for presumably the milk and bread (*i.e.* lactose and dextrin) received by these patients was sufficient for their needs in keeping excessive numbers of proteolytic organisms in check. The diet given to these patients would therefore seem to have been satisfactory.

Toxaemia in severe cases would appear to indicate the need for examination of the stools for putrefactive organisms, and, if these are present, treatment with increased carbohydrate diet might be advisable, but in the milder cases the diet given on similar lines to that prescribed for these four cases would appear to be satisfactory.

#### SUMMARY OF CONCLUSIONS.

1. The appearance and disappearance of typhoid bacilli in the faeces in these four cases bore no relation to changes in the diet nor to the physical state of the stools, but the later they appeared the shorter was the duration of their appearance.

On the disappearance of typhoid bacilli from the stools the intestinal flora tended to become more simple.

2. *B. coli* was the only organism invariably present at every examination.

3. Streptococci were very much more abundant in the earlier stages of the disease, when milk formed the greater part of the diet, than in the later stages.

4. In two cases where boils occurred on the body the causative organism had previously been isolated in large numbers from the faeces.

5. With stools slightly alkaline to litmus the flora in these cases was relatively simple and fermentative in type. There is no apparent advantage, therefore, in giving a high carbohydrate diet except in cases of marked alkalinity and putrefaction.

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## A REPORT ON PLAGUE INVESTIGATIONS IN EGYPT.

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(With 1 Map and 2 Charts.)

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## A SURVEY OF THE HISTORY OF PLAGUE IN EGYPT.

*The Physiographical Features and the Population of Egypt.*

THE two generally recognized divisions of the country are the Delta (Lower Egypt), extending from the Mediterranean littoral to Cairo; and Upper Egypt, the narrow strip of alluvial soil in the Nile Valley from Cairo to Wadi Halfa. The valley of the Nile is a rift valley formed by the subsidence of a narrow belt in the neighbourhood of a line of fracture on the earth's surface; from Cairo to Aswân town its length is 880 kilometres (547 miles), and its width varies from 3 to 21 kilometres (2 to 13 miles). The province of the Faiyûm is a depression which is irrigated from the Nile by the Bahr Yûsef Canal, one of the old natural drainage channels of the valley.

There are three climatic regions in Egypt: (1) the northern coast and the Delta; (2) northern Upper Egypt from Cairo to Asyût; and (3) southern Upper Egypt from Asyût to Wadi Halfa. The climate of the first region is determined by conditions existing over the Mediterranean; that of the third is independent of these conditions; and the second forms a transition region, which is governed to some extent by Mediterranean influences but chiefly by its proximity to the desert. Upper Egypt has a hot dry climate tempered by the prevailing wind, which blows from the north. The *khamsein* lasting for a few days in spring

<sup>1</sup> The account that follows has been abridged from the authors' original report of work which they carried out from November, 1911, to November, 1913, on behalf of the Department of Public Health of the Egyptian Government (*Report No. 5 of the Public Health Laboratories, Cairo*. Govt. Press, 1923). Therein will be found the full protocols of the experiments. For the purpose of this inquiry the services of Dr G. F. Petrie were lent by the governing body of the Lister Institute.



is a warm, oppressive, sand-laden wind from the south. With the exception of a belt near the Mediterranean the country is almost rainless. The Nile flood reaches Egypt in the month of July, and is furnished mainly by the monsoon rainfall on the tableland of Abyssinia.

The report of the Census taken in 1907 gives the total population of the cultivated areas (excluding nomad Arabs) as 11,189,978, of which Upper Egypt contributed about five millions. The density of population was 939 persons per square mile (362 per square kilometre); Aswân District had the highest density, namely, 640 per square kilometre. There were 3581 villages, most of them with less than 10,000 inhabitants; the greater part of the population is aggregated within these villages.

*The History of Plague in Egypt previous to 1899, the Year  
of its Reintroduction.*

Egypt has been frequently represented in the literature as one of the nurseries of plague in the world. There is little doubt that formerly it was often attacked by the disease, and that the old epidemics were of far greater intensity than those of the present day. Scattered throughout the chronicles to the year 1844, there are references to at least 121 years of plague prevalence. Cairo is singled out as suffering from disastrous epidemics; twenty-five notable outbreaks from 1142, the date of the first recorded epidemic in the city, to the year 1841, have been described. The figures for some of them betoken an appalling mortality; for example, it is said that there were 860,000 deaths in the years 1574-6 (Sticker) and 500,000 deaths in the epidemic of 1581 (Prosper Alpinus). The older estimates must, however, be taken with much reserve. Patrick Russel (1791) was sceptical of the accuracy of the contemporary Egyptian figures, and Aubert-Roche (1843) drew attention to the manipulation of the statistics that were compiled for the plague epidemics of the years 1834-5 in Alexandria. One source of fallacy, besides the tendency to Oriental exaggeration, is the concomitance of plague and of famine due to exceptionally low Nile floods; the deaths from these causes must often have been wrongly assigned. The most convincing records that we have seen are those of Gaetani, which give the number of deaths for each day in the Cairo epidemic of 1835; the total is 33,751 for six months. But even if statistical over-statement be admitted it is none the less certain that Cairo was wont to be severely smitten by plague; and its present complete immunity is the more remarkable.

Apart from numerical data the old literature of plague in Egypt consists largely of speculations on the origin and predisposing causes of the disease. We have failed to discover in the early writings specific reference to an associated mortality amongst rats or any evidence that pneumonic plague was recognized as a distinct variety of the disease.

The history of plague in Egypt may be conveniently divided into three periods. The first ends in the year 1798, when the modern study of the disease began with the observations

of the medical officers who served under Napoleon with the French Expeditionary Force. The second period extends from 1798 to 1899, the year of the reintroduction of plague into the country. The third is concerned with the recent epidemics.

Prior to the seventh century the records are scanty and of little value. The manuscripts of a number of Arabic scholars, who described plague epidemics that occurred in Egypt from the seventh to the close of the fifteenth century, were examined and abstracted by von Kremer (1880), the Viennese orientalist, in his monograph "On the Great Epidemics of the East collated from Arabic Sources." The principal author whom he cites is Sojuty; his rare treatise deals exclusively with plague, and was discovered by von Kremer in Cairo. Sojuty brings together the great epidemics from the beginning of Islam (622 A.D.) to the year 1492, the date of his treatise. Abd Allatif, the celebrated Baghdad physician, observed the devastating plague and famine of the year 1200, and has left a graphic account of the widespread distress that ensued. The Arabic traveller from Tangier, Ibn Batuta, experienced an outbreak of plague in India on his return from China, fell ill with the disease in 1332 at Muttra near Agra, and encountered an offshoot of the Black Death pandemic (1346-52) in the summer of 1348 in Cairo. The epidemic of 1581 was described by Prosper Alpinus, the physician to the Venetian Embassy in Cairo; he specially noted the regular seasonal recurrence and decline of the disease.

The first sustained and concerted efforts to investigate the problems of plague were made early in the nineteenth century in Egypt. The French and Russian Commissions, which worked at intervals between the years 1828 and 1843, had the distinction of being the first to be expressly appointed for this purpose. In 1828 the French Government sent a Commission to study the disease, and in 1835 the better known Commission headed by Clot Bey—the other members being Gaetani Bey, Lachèze, and Bulard—worked for seven months in Cairo and Alexandria, and excited a lively controversy on the question whether plague is or is not contagious; except Bulard they were firmly persuaded that it is not transferable. Clot Bey considered the possibility of a causal microbe, for he mentions the belief of Father Kircher (1658) that in the course of an epidemic at Rome he had seen the infecting agent of plague with the aid of a microscope. Further evidence of Clot's acquaintance with the scanty references to this aspect of the subject is shown by his quoting from Manget (1721) an account of observations, which appear to us to be a forecast of later knowledge, because they indicate a connexion between the prevalence of insects and plague, and even recall the method in use at the present day for establishing the correlation. Manget states that during an epidemic in 1712 at Copenhagen an observer noted that the injurious effects of a plant disease, which was caused by a minute insect, became worse as plague spread over the city; he had discovered the insects by means of a microscope, and he actually demonstrated the increase in their numbers by collecting them on sheets of white paper, which were exposed overnight in the open air. Clot Bey was apparently not content with the information available to him, since he tested the infectivity of plague materials on his own person. He inoculated himself on two occasions, with the blood of a patient and again with pus from a bubo; he also inoculated various animals. All his experiments were negative, and it is perhaps not to be wondered at that he rejected the hypothesis of a microbic origin of the disease.

In February 1843 a Russian Commission (Uratichco and Ischernikoff) experimented in the Qasr el 'Aini hospital in Cairo on the disinfection by heat of infected articles of clothing. They took the clothing and bedding of patients, and heated them at a temperature of 62° C. to 75° C. for 48 hours. The clothing was given to natives, who wore it for fourteen days and took no harm; some of the heated clothes were sent to Odessa, and were worn there by twenty persons; they, too, remained healthy. Forty-nine persons, including doctors and hospital attendants, who nursed the patients or slept in the wards or carried the clothing to the disinfection stoves in Cairo, escaped ill effects.

The experiments of this period, although inconclusive, are interesting, for they anticipate

the accurate observations that were made possible by the discovery in 1894 of the *B. pestis*. In the year 1844 plague disappeared from the country (Prus); the free period lasted for fifty-five years, until the reappearance of the disease in 1899 in Alexandria.

*The Origin, Diffusion, and Significant Features of the  
Series of Epidemics, 1899-1911.*

In April 1899 plague broke out in Alexandria. The exact mode of its importation is unknown, and is of little consequence; for it is indisputable that Egypt became infected in the expansion of the current pandemic, which spread from Hong Kong in 1894 and entered India in August 1896 through the port of Bombay. In 1897 Jeddah, on the coast of the Red Sea, was officially declared to be infected; a second outbreak occurred here in 1898, and a third in February 1899. This town may have been a stage on the route of infection from India to Egypt.

The outbreak in 1899 in Alexandria numbered ninety-three cases and lasted from April until November; the rest of the country remained free. In 1900, 127 cases were notified, and the disease was confined to Port Said, Alexandria, and Damietta, the ports of the Delta. In 1901, 200 cases were reported altogether from Alexandria, Port Said, and several provinces of the Delta; and in this year also the first appearance of the disease in Upper Egypt was signaled by two small outbreaks in Minya District. In 1902 plague became widespread in the Delta, and exhibited a definite infiltration of Upper Egypt, where it settled upon the provinces of Minya, Asyût, and Qena. The subsequent history is one of a generalized dispersion of the infection throughout the country. From the date of the introduction of plague in 1899 up to the end of the year 1912, 10,007 cases were notified; the total number to the end of the year 1919, a period of twenty years, is 14,783 cases; all the clinical forms of the disease are included in these figures. There has been no apparent change in the character of the epidemics since the close of our work.

When we began our work several unexplained features of plague in Egypt presented themselves. There is, as in most places, a definite seasonal prevalence, but the disease does not conform to a single epidemic wave flowing and receding over the country; because the epidemic months fall in the first quarter of the year in Upper Egypt and during the summer months in the Delta. The singular tendency for outbreaks of pneumonic plague to appear in certain of the Upper Egyptian provinces, although well known to the Health authorities, had defied explanation and had escaped the notice of commentators on plague. Again, the local circumstances that accounted for the exceptional severity of the Kôm Ombo outbreak in 1911 were not wholly understood. Lastly, it was noteworthy that the infection had failed to take root in Cairo. This city, placed at the apex of the Delta where it joins Upper Egypt, necessarily receives the streams of traffic which pass constantly to and from these regions; and it is, therefore, not surprising that Cairo has incurred the risk of the importation into it of bubonic plague.

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### THE SPECIES OF RODENTS TRAPPED IN THE HOUSES, FELUCCAS, AND CULTIVATED AREAS OF UPPER EGYPT.

#### *The Rodent Infestation of Houses, Feluccas and Cultivated Areas.*

The general results obtained by trapping various localities are summarized in Table I, which shows the regional distribution and relative frequency of 67,000 rodents captured from houses by setting 233,601 traps. *R. rattus* heads the list (nearly 40,000), *Acomys cahirinus*, the so-called Cairo spiny mouse, taking second place (25,000). *Arvicanthis niloticus*, the common field rat, and *R. norvegicus* only occasionally frequent houses. The kind of trap used was not suitable for catching mice, but there was little evidence that they inhabited buildings. Five shrews (*Crociodura olivieri*), three jerboas (*Gerbillus pyramidum*) and seven weasels (*Mustela africana*) were also captured.

Table I.

(Reduced from the original table) showing species and numbers of rodents trapped from houses in various localities in Upper Egypt.

Locality		Species and numbers of rodents trapped							
Towns and villages	Province	<i>R. rattus</i>	<i>Acomys cahirinus</i>	<i>Arvicanthis niloticus</i>	<i>R. norvegicus</i>	Mouse	Weasel	Shrew	Jerboa
Cairo ... ..	—	88	129	—	—	1	7	—	—
2 villages ... ..	Minia	21	6	—	—	—	—	—	—
Asyût town ... ..	Asyût	25231	19328	634	566	—	—	1	—
17 villages or towns	„	12320	1942	32	62	3	—	1	—
2 villages ... ..	Girga	315	1058	41	—	1	—	3	—
Qûs town and 5 neighbouring villages	Qena	1944	2743	79	—	19	—	—	—
Ibrahim Aly's hut near Sharany	„	—	—	3	54	—	—	—	—
Kôm Ombo ... ..	Aswân	—	—	243	37	92	—	—	3
Totals:		39919	25206	1032	719	116	7	5	3

*R. rattus*. The breeding of *R. rattus* in Asyût town is at its height in the summer months, when plague prevails in the district; at this time of the year 40-50 per cent. of the adult females were pregnant. The average number of foetuses in 2226 pregnant rats was 5.8.

In our experience *R. rattus* has a widespread distribution over Upper Egypt. 19 per cent. of the rodents trapped from feluccas (native river-craft) were of this species.

*Acomys cahirinus*. The wide distribution of this rodent in the houses of Upper Egypt had not been previously recognized, although in some localities it exceeds *R. rattus* in number. It lives in cracks and crannies in the walls of the houses. In Asyût town the breeding season is the same as that of *R. rattus*; 3312 pregnant *Acomys* gave an average of 2.5 foetuses. In the summer months 80–90 per cent. of the trapped females were pregnant, from which it is clear that the species is very prolific. 8 per cent. of the rodents trapped from feluccas were *Acomys*.

*R. norvegicus*. During two years 682 *R. norvegicus* were caught; 566 were from Asyût town, and 343 of these were captured in corn-mills. The invasion of the town by this species is correlated with the fact that it is the principal rodent inhabiting the feluccas. The following experience indicates that, in places remote from towns and villages, scattered colonies exist, and that they are founded by migrants from feluccas. 54 *R. norvegicus* were caught in and around a single-roomed *boos* (dried millet stalks) hut, which stood alone on cultivated ground quite close to the Nile and half a mile from the village of Sharany (Qena Province). The interior arrangements of the hut were such as to provide food and shelter for rats. There were rat holes at the base of the dried mud grain receptacles (*somâs*) and beneath the straw palisades that formed the walls of the hut.

Observations on the rodent infestation of the Delta collected by Dr C. Todd prove that in the Delta *R. norvegicus* is more widely spread and forms a larger part of the rat population than in Upper Egypt. Feluccas ply along the two branches of the Nile from Rosetta and Damietta to Cairo and thence to Aswân, and they doubtless help to distribute this species throughout Upper Egypt; the railways are also a likely means of conveyance. 72 per cent. of the rodents caught in feluccas were *R. norvegicus*. Excepting the Kôm Ombo estate this rat was never trapped in cultivated fields.

*Arvicanthus niloticus*. Apart from the figures for the Kôm Ombo estate 789 were caught in houses, and of these 634 were from Asyût town. Here the majority were trapped in about equal numbers from corn-mills and living-places. This rat is seen everywhere in the open country. After the crops are harvested it lives in the deep cracks in the sun-baked soil, from which it is driven out when irrigation commences.

### *The Flea Infestation of Egyptian Rodents.*

*X. cheopis* contributed from 90–100 per cent. of the fleas from *R. rattus*, *R. norvegicus*, *Acomys*, and *Arvicanthus* trapped in houses.

*R. rattus*, *R. norvegicus*, and *Acomys*, when trapped from the feluccas during the cool season, harboured *Leptopsylla musculi* in addition to *X. cheopis*; the former species was sometimes the predominating one. Three specimens of *Ceratophyllus fasciatus* were taken from *R. norvegicus* trapped on feluccas at Asyût town. *Acomys* from Cairo had a marked infestation of *X. chephrenis*; a specimen of *Gerbillus pyramidum* from Kôm Ombo gave a high percentage of *X. cleopatrae*; on a hedgehog (*Hemiechinus auritus*) most of the fleas were *Ctenocephalus felis*; and a weasel (*Mustela africana*) carried *Echidnophaga gallinaceus*.

The figures for Asyût town exhibit a definite seasonal wave for both *R. rattus* and *Acomys* with a maximum for each species in March (1912); in this month the average number of fleas was 11.1 on *R. rattus* and 1.2 on *Acomys*. The flea-curves for both years correspond well with the epidemic curve for plague in the district.

*Acomys* harbours only about one-tenth the number of fleas that are carried by *R. rattus*. For this reason, though susceptible to plague, it is of little importance in spreading the disease; we never discovered a naturally infected *Acomys*.

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OBSERVATIONS IN UPPER EGYPT ON THE RANGE OF EXCURSION OF THE  
 HOUSE RODENTS: *R. RATTUS* AND *ACOMYS CAHIRINUS*.

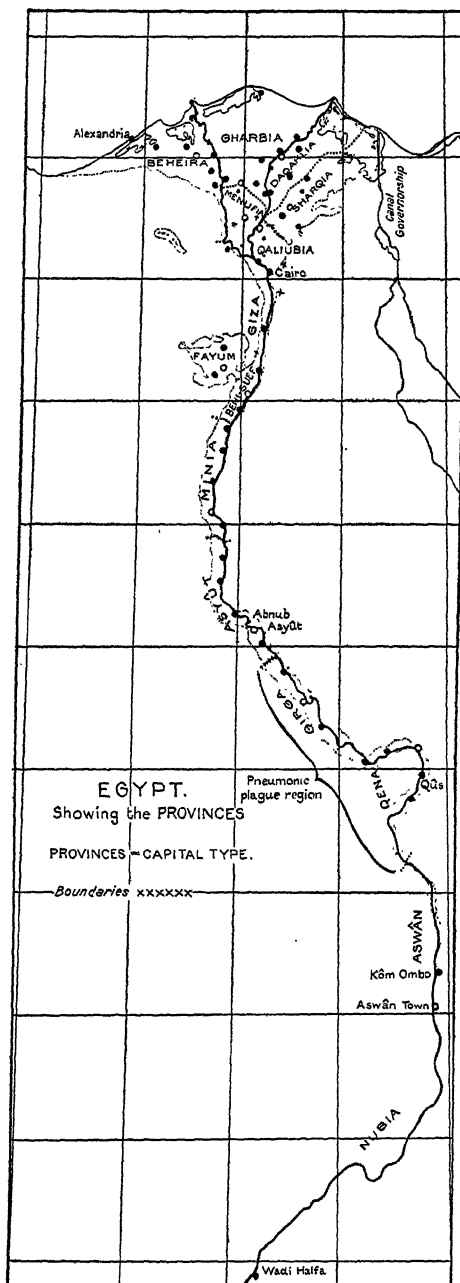
After several months' experience of plague-infected localities in Upper Egypt, it became evident to us that here, as in other countries, rats, and especially *R. rattus*, are active propagators of the infection. Now the spread of the epizootic and consequently of the epidemic must be influenced by the movements of rats from house to house, and by more distant excursions if they occur. We could not find that any attempt had been made elsewhere to ascertain the normal movements of *R. rattus* living in human habitations, although dubious statements were on record of migrations of this species in considerable numbers from plague-stricken areas in India. Accordingly, having become familiar with the habits and environment of *R. rattus* and *Acomys*, we sought to learn the range of movement that is characteristic of each of those species.

*Observations in El Motiâ.*

The experiment that we carried out here lasted from March 23 to June 21, 1913, a period corresponding to the local plague season. El Motiâ (Asyût Province) is a typical Upper Egyptian village, and is situated on the Nile bank eight miles south of Asyût town.

Maps of the portion under experiment, indicating each house with its number, were prepared. A constant number of traps (300) were set daily, two traps being set in each house. They were collected next morning, and each animal captured was marked and put back into the house from which it was caught. 450 traps were also set every day in the surrounding squares, and the resulting takes were examined for marked rodents; all the animals caught from this area were returned unmarked to the houses whence they came. Entries of all the rats that were captured, marked, and recaptured were made in a carefully tabulated register. By means of devices used for marking the rats it was possible to identify animals from every one of the 279 houses in the area. At first it was thought sufficient to identify the house from which the rodents were originally captured, but by an extended system of cautery marks made on various regions of the body at each successive capture many individual rats could be recognized.

From the results summarized in Table II we conclude that the movements of *R. rattus* and *Acomys* are limited; most of them are oscillations between contiguous houses, and doubtless represent sorties in quest of food from nests





and burrows situated in the foundation of walls that were common to adjoining houses.

Table II.

*Summary of trappings, captures, and movements of marked rodents in the experimental square, El Motiâ.*

	<i>R. rattus</i>	<i>Acomys calhirus</i>
No. caught and marked in the square ... ..	341	348
Casualties amongst these ... ..	36	79
No. of marked rodents returned to houses ... ..	305	269
"   "   "   not again captured ... ..	159	122
"   "   "   recaptured one or more times ... ..	146	147
"   "   "   caught in more than one house ... ..	53	46
"   movements to and from contiguous houses separated by a party-wall*	68	40
"   excursions of greater range within the square† ... ..	13	12
"   excursions to neighbouring square... ..	1	0
"   trappings to which recaptured rodents (146 <i>R. rattus</i> and 147 <i>Acomys</i> ) were exposed‡	2458	2685
"   captures of recaptured rodents ... ..	436	390
Ratio of captures to trappings of recaptured rodents ... ..	1 : 4.1	1 : 5.2
"   movements to captures of recaptured rodents... ..	1 : 6.7	1 : 9.3

\* Each oscillation of a to and fro movement has been regarded as a separate movement.

† The stages of these excursions have not been counted separately.

‡ This figure was arrived at as follows: For each rodent captured more than once the number of the last trapping at which it was caught gives the number of its exposures to trapping; hence the total number of exposures to trapping for all the recaptured rodents was obtained by summing the last trapping number of each.

The speculation that, when plague breaks out in a colony of *R. rattus*, some of the rats take fright, desert the colony, and so help to spread the infection, is not borne out by our observations in El Motiâ. Both *R. rattus* and *Acomys* seemed to us to be singularly little impressed by unpleasant experiences, an opinion confirmed by analysing the frequency of captures of individual rodents. There is no ground for the belief that rats are conscious by instinct or reasoning of the risk of contracting a fatal disease from their sick companions or that they are alarmed by an unusual mortality in the colony. Proof of the peculiarities of rodent psychology can with difficulty be conveyed to others apart from actual demonstration; but we are sure that the movements of rats are not increased by motives of fear when an epizootic is in progress. Unfortunately a migration experiment in a town or village where plague is epidemic is not likely to succeed owing to hindrances that may be expected from the heightened suspicions of the people.

#### OBSERVATIONS ON THE RELATION BETWEEN EPIZOOTIC AND EPIDEMIC PLAGUE IN UPPER EGYPT.

We specially observed three localities where human plague was associated with plague in rodents, namely: Abnûb town (Asyût Province), Qûs town (Qena Province), and Kôm Ombo (Aswân Province).

Our investigation of these outbreaks revealed individual differences which were of such a degree as to justify their separation into epidemic types. Thus the outbreak, in both rats and man, in Abnûb town, is representative of plague as it may be observed elsewhere in the northern provinces of Upper Egypt;

it resembled in essentials the town and village epidemics that prevail throughout the East. The epizootic in Qûs town was of the normal type, but the characteristic feature of the epidemic was the interspersing of foci of pneumonic plague amongst bubonic cases; this outbreak may be regarded as a pattern for the southern provinces of Upper Egypt. The large agricultural estate of Kôm Ombo, situated in Southern Upper Egypt, is however an altogether exceptional locality. For the hamlets on this estate do not possess the traditional configuration and structural arrangement of Upper Egyptian villages; and in relation to these differences the circumstances that favoured the spread of plague exhibited peculiarities that concerned both the epizootic and the epidemic.

*The Epidemic of Bubonic Plague in Abnûb Town*  
(January to April 1912).

In a population of 16,000 persons 48 cases, all of which were bubonic or septicaemic, were recognized. We made observations in the first infected area, and obtained proof of the general concurrence in time and place of the epizootic and the epidemic. Experiments with rat-fleas caught in this area are as follows:

(1) 295 fleas were caught at 12 noon, January 8, from seven *R. rattus* trapped together; three of the rats were plague-infected. The fleas were transferred to the laboratory at Asyût and were received there at 5 p.m. on January 11. At 7 p.m. on this date, seventy-nine hours after removal from their hosts, 150 fleas were put with a guinea-pig in a flea-proof cage. The guinea-pig died of plague on January 18, and the post-mortem signs included left submaxillary, left axillary, and left inguinal buboes, which contained numerous *B. pestis*.

(2) The rats from which the fleas of the previous experiment were taken had been trapped in a very dirty store-room, which opened into a stable; above the store-room there was a room filled with grain. On January 9, a guinea-pig was allowed to run free in the store-room. Next morning it was searched under chloroform, and thirty-six rat-fleas were taken from it. On January 14, the guinea-pig died of plague, and showed submaxillary and left inguinal buboes in which numerous plague bacilli were found.

(3) About thirty fleas caught at 1.30 p.m. on January 10, from the guinea-pig in the foregoing experiment, were transferred to the Asyût laboratory, where they were received at 5 p.m. on that day. They were kept unfed in a test-tube till January 12, when they were put with a guinea-pig in a flea-proof cage forty-eight hours after their removal from the animal that served as a flea-trap. On January 19, the laboratory guinea-pig was chloroformed to death; it presented right and left submaxillary buboes, which contained the *B. pestis*.

*The Epidemic of Bubonic Plague in Qûs Town*  
(January to April 1912).

Of a total of eighty-two cases that were notified from a population of 15,000 persons, fifty-one were bubonic, sixteen had no obvious buboes, and fifteen (18.3 per cent.) were of the pneumonic form. There were in all sixty-one deaths (74.4 per cent.). The distribution of the bubonic cases was so irregular as to suggest the existence of at least four isolated epizootic areas; and a spot map which we prepared shows very well the disorderly arrangement of the

outbreak. We are of opinion that the creation of apparently independent foci of rat and human plague in a town like Qûs is attributable to the transportation of infective rat-fleas by persons who inhabit or visit infected areas in the town; these persons may or may not suffer from plague in consequence. In this outbreak some of the patients were suspected of having contracted the infection in a distant quarter of the town.

14,000 traps were set over the whole town from March 13 to June 19, and they captured 1622 *R. rattus* and 2566 *Acomys*. The number of rats with the signs of acute plague (11) that were found was small, chiefly because the people gave us no help in collecting them. Towards the end of the outbreak 11 rats trapped alive had definite or presumed resolving (chronic) plague lesions.

We were fortunate in having been able to make a thorough examination of a badly infected house in Qûs, which had been vacated voluntarily by its owner. The house, which was inhabited by a wealthy Coptic family, was temporarily abandoned early in April on account of plague infection. On April 11 the routine cleansing of the house, including the removal of dead rats, the closure of rat holes, and disinfection with "sublimite" solution, was performed by the Public Health authorities. Our examination was begun on April 21, 1912.

The building is a typical Upper Egyptian dwelling-house of burnt bricks with an upper floor and flat roof; it encloses three sides of a courtyard, which is bounded on the remaining side by the wall of an adjacent house. Five rooms open into the courtyard from the ground-floor; one of them is used as a store-room for fodder and another as a stable for donkeys and domestic animals. The living-rooms on the upper floor are reached by a brick staircase and are entered from balconies built over the courtyard. On the flat roof there are two small rooms. The floors of the upper rooms and balconies consist of a double layer of burnt bricks bound together with clay; these are supported by the lath-like stems of palm-tree leaves, which are laid across stout beams made of split palm-tree trunks. At our first visit, on April 21, we noted the distinctive smell of decomposing rats. A sick *R. rattus*, shown later to be plague-infected, was seen crouching near a wall of the courtyard.

The cesspit shaft was the chief focus of infection; it was discovered as follows. Facing the entrance to the courtyard there was an archway loosely filled with bricks. By their removal a narrow passage little more than the breadth of a man was disclosed; it led round three sides of a rectangular brick shaft, the remaining side of the shaft being formed by the outer wall of the house; a sick rat was observed to retreat through a crack between the bricks of the shaft. The floor of the narrow passage was littered with rubbish and with dead rats, of which thirty were afterwards removed. In a small room on the upper floor immediately above the cesspit there was the usual kind of native privy; a dead infected rat lay near the opening of the privy, and rat burrows tracked into and around the cesspit shaft. The burrows were partly exposed by clearing away the bricks round the privy, and six dead rats were disinterred.

A guinea-pig placed on April 22 in the space at the base of the cesspit shaft and kept there for three days collected 684 rat-fleas, and died of plague on April 25. 559 fleas taken from this animal were transferred to a healthy guinea-pig in a flea-proof cage; the latter died of plague on April 28. We proceeded on June 4 to demolish the cesspit shaft, and in doing so opened up many rat burrows that communicated freely along its whole length. The walls of the shaft were composed of bricks held together loosely with dried clay. Fifty-three dead *R. rattus*, one *Acomys*, and many scorpions were taken from it during its demolition.

A recess underneath the brick staircase that led to the upper floor had been walled off except for a small doorway closed loosely with bricks. The recess was explored, and a dead *R. rattus* was seen on the floor; it was found to be plague-infected.

The loose nature of the flooring was made apparent by dislodging the bricks round several of the rat holes and burrows in the balconies. The clay between the bricks had become pulverized so as to form spaces, which contained many dead rats. The junction of the walls in the rooms showed gaping fissures, some of which ran from floor to roof and afforded good foothold for climbing rodents. Dead rats were picked up from the floor of most of the rooms, having fallen presumably from the beams of the ceilings.

In each of eight rooms a guinea-pig was isolated; five of them died of plague (three in ground-floor rooms and two in upper rooms), two kept healthy, and one was lost. Guinea-pigs placed in the two rooms on the flat roof did not catch any fleas and remained healthy. There was no evidence of rat infestation here, probably because the relative thinness of the walls gave no shelter for rats. Two of our workmen, one of whom unfortunately died, contracted plague in the house during its examination.

*Plague on the Kôm Ombo Estate*  
(1911 and 1912).

The Kôm Ombo agricultural estate was founded about the year 1900 and is situated on the Nile bank 836 kilometres (520 miles) south of Cairo and 48 kilometres (30 miles) north of Aswân town. Scattered over the estate there are twenty-nine hamlets (*ezbas*), with a total population in 1912 of 12,831, and an average of 250 persons. The inhabitants are migrants from the southern provinces of Upper Egypt. By providing work all the year round, the Kôm Ombo estate is a centre for casual labour, and on account of its recent origin the settlers do not constitute a fixed population.

In January 1911 plague appeared on the estate for the first time. Many of the hamlets became infected, and the spread of the infection was so rapid that hundreds of the people fled in panic to their native districts, and left some of the villages almost deserted. 357 plague cases with 237 deaths were recorded from January to the end of April, but the figures are certainly an under-estimate.

In November 1911 we visited Kôm Ombo, which was then free from plague, and made observations on the rodent infestation of the villages and of the cultivated land. In the last week of January plague broke out at El Abbasiya (population 920 in 1912), a hamlet in the south-east corner. The next hamlet to be attacked was Atmour Mistigid (population 364 in 1912), situated in the north-east corner; the first of a total of twenty cases occurred on February 7. Shortly after plague broke out in this village the inhabitants, taking their animals with them, abandoned it at the instance of the Public Health authorities, and were accommodated in a temporary camp of matting huts a little distance away. We were then able to make a complete examination in advantageous circumstances of the plague conditions of Atmour Mistigid.

This hamlet was less than a year old in February 1912, for it had been built since the epidemic in Kôm Ombo of the previous year. It comprises about 100 mud houses, which are arranged in four blocks. Two of the blocks, each consisting of two rows of back-to-back houses, are in alignment, and are separated by an opening for traffic. On the opposite side of the street two rows of "single" houses are similarly arranged. The plague patients had resided in the back-to-back houses, and in these most of our experiments were made.

The huts are of the simplest construction; each consists of a single room six feet square, with mud floor and walls and with a straw roof supported by a wooden pole. Most of the owners had added a small courtyard, in which to stable the larger domestic animals; these were enclosed by straw palisades, which were cleared away when the villagers betook themselves to the temporary camp.

On February 12 the Public Health authorities cleaned the houses and treated them freely with perchloride of mercury solution (1 in 1000); at the same time the straw roofs were removed, and thus the walls and floors were exposed during the day to the brilliant and continuous sunlight. Our experiments were begun three days later.

We first trapped the empty village but did not capture any rats, nor were any dead ones seen. This result was puzzling in view of the considerable number of human plague cases, and the capture of *Arvicanthis niloticus* and *R. norvegicus* in similar but uninfected houses elsewhere on the estate. As the sequel indicates, the absence of rats was due to the heavy mortality amongst them from plague, and perhaps also to the desertion of the survivors owing to the removal of food from the huts.

The next step was to allow a guinea-pig to run free for twenty-four hours in the houses, including those in which the plague patients had lived. The results from twenty-seven houses showed that numerous rat-fleas (*X. cheopis*) were caught on the guinea-pigs that served as traps; the extreme figures were 15 and 1005, and fourteen of the houses yielded 120 or more rat-fleas. Four of the houses that had been occupied by plague-stricken families were proved by the death of the guinea-pigs from the disease to be still infective: experiments were carried out later in these houses to determine the period of infectivity; and from the results obtained we conclude that the infection persisted in them for nearly a month. The extraordinary flea infestation of the houses deserves notice; we have never seen any record of observations at all approaching our experience here.

Attempts were made to rid the houses of fleas by treating them with substances recommended as flea-killing agents, for example, cyllin or other coal-tar derivatives and petroleum emulsions. The primitive construction of the houses permitted a thorough application of these fluids, but the results were invariably disappointing. When we consider the far more complicated structure of Upper Egyptian houses, we find it difficult to believe that substances of the kind used can do more than a minimal amount of good in suppressing or even materially reducing plague infection within them.

Our attention was next directed towards discovering the source of the rat-fleas in the houses. This was not obvious, because we could see no plain signs of rat holes or rat burrows except the cracks at the junctions where four walls of the back-to-back houses meet. The experiments designed to elucidate the point were as follows:

Guinea-pigs remained free from fleas when they were confined to shelves placed along the walls at a height of one foot and upwards above the ground-level. Further, rat-fleas could be partly excluded from the floor of the houses, if the bottom of the walls was sealed with wet mud. Lastly, in a house which had previously yielded 2843 rat-fleas in all on guinea-pigs introduced as traps, a guinea-pig was allowed to run free for twenty-four hours; it caught 208 rat-fleas. Three days later a fresh animal was confined for twenty-four hours

in a rat-trap placed at the centre of the floor; it caught only fifteen fleas. Two days later thirty-five Tanglefoot papers were arranged side by side along the junction of the walls and floor, and kept there for twenty-four hours; during this time a guinea-pig was allowed to run free on the floor. 402 fleas were caught in the Tanglefoot and only twenty-eight on the animal let loose on the floor. All the papers except six had one flea or more, and 105 was the largest number on a single paper.

These observations taken together made it apparent that the fleas must have issued from the inconspicuous fissures at the junction of the walls and floor, and from no other part of the house. When the houses had lost their infectivity, two of them were broken down—with difficulty on account of the surprising coherence of dried Nile mud—and the rat burrows were exposed and traced. A regular system of nests and burrows existed at the bottom of the walls with free communication at their junctions, an arrangement that indicated continuity along the whole length of each block of houses. Mummified *R. norvegicus* and *Arvicanthus niloticus* were removed from the nests and burrows; the number of rat-fleas found here was remarkable, and clearly pointed to the nests and burrows as the breeding place and depot of the fleas that infested the houses. The failure of the attempts to rid the houses of fleas by substances that are ordinarily lethal to them through contact for a sufficient length of time is explained by the inaccessible retreat of the fleas in the foundation of the thick mud walls.

*Arvicanthus*, *R. norvegicus*, and mice infest both stone and mud-built houses and also the cultivated fields. The absence of *R. rattus* and *Acomys* is easily explained, for it is unlikely that those species could compete successfully with *R. norvegicus* and *Arvicanthus* in the kind of houses we have described. The continuous irrigation throughout the year must interfere with the settlement of rat colonies on the land. Certainly the density of rodents was greater in the hamlets, where the conditions gave them every encouragement. The houses serve the double function of dwelling-place and animal stable, a combination frequently to be seen in Upper Egypt. Goats, donkeys, sheep, and fowls share them with their owners; and camels, oxen, and buffaloes are tethered in the enclosures annexed to each house. Grain and other foodstuffs are either stored in receptacles made of dried mud and thus penetrable by rats, or they are wholly unprotected.

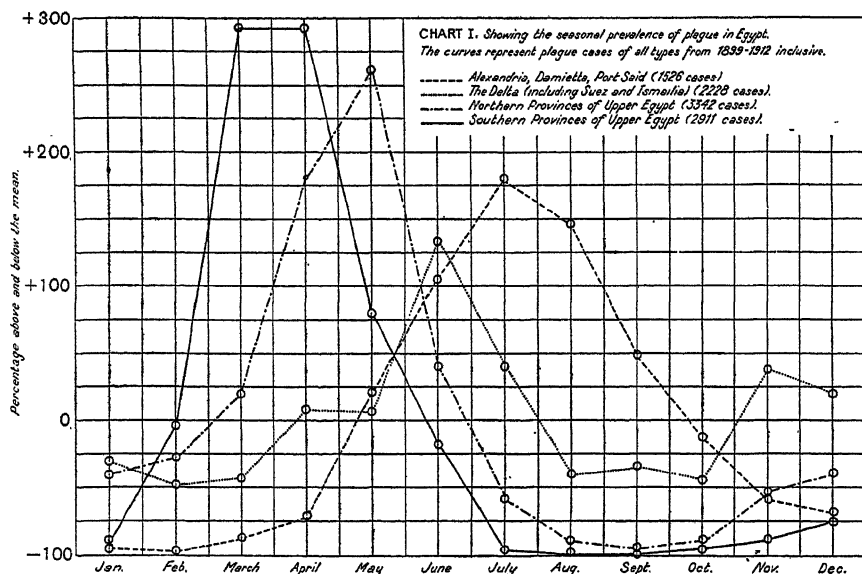
The possibility of the carriage from one hamlet to another of infected fleas by human beings is strengthened by the results of examining the clothes of four plague patients from Atmour Mistigid. Only two *P. irritans* were found in the four lots of clothes, and the numbers of *X. cheopis* were 4, 4, 4, and 1; one of the sets of clothes belonged to Ahmed Mohammed Saad who, when detected, had walked five miles in order to escape quarantine. In contrast with these results thirty-two lots of clothing from healthy persons did not contain any rat-fleas; they yielded only three human fleas. And, further, 185 *X. cheopis* were taken from the clothes of three of our rat-catchers after they had dug out nests in houses in Atmour Mistigid.

The exceptional severity of the epidemic in the year 1911 is adequately explained by our observations. Foremost must be placed the quite remarkable

degree of infestation of the houses with *X. cheopis*. With the exception of a few *P. irritans* this was the only species of flea found. Bugs were never seen. We have never observed and cannot imagine conditions more favourable to the spread of bubonic plague than those which exist in the mud-built villages on this estate. The back-to-back arrangement of mud houses in a continuous row is an ideal one for facilitating the intercommunication of rat colonies, the dispersion of epizootic plague, and the transference of infected rat-fleas to the human occupants.

#### THE SEASONAL PREVALENCE OF BUBONIC PLAGUE AND ITS RELATION TO CLIMATE.

Plague in Egypt, as elsewhere, prevails within well-defined time-limits for each infected locality. The monthly figures for plague cases from 1899 to 1912 have been grouped and charted to correspond with four divisions of the country: (1) Southern Upper Egypt; (2) Northern Upper Egypt; (3) the provinces of the Delta; and (4) the northern ports on the Mediterranean, namely, Alexandria, Damietta, and Port Said (Chart I). The chart is composed of a regular succession of epidemic waves associated with an increasing delay in the seasonal prevalence of the disease. Thus the maximum prevalence for Southern Upper Egypt is in March–April; for Northern Upper Egypt in May; for the Delta in June; and for the northern ports in July. The rise of the epidemic in the Delta is roughly synchronous with the decline of the epidemic in Upper Egypt.



It is a natural supposition that the varying climatic conditions afford a clue to the characteristic delay in the seasonal prevalence from south to north; but when we came to examine the subject, difficulties arose which were due chiefly to the smallness of the plague

data even when they were massed for the longest available period. Brownlee (1918) has pointed out that the best materials for study are obtainable from populous cities with a continuous plague history of several years that furnish considerable numbers of cases, for example, some hundreds or even thousands annually. The Egyptian statistics being excluded by adopting this standard as a measure of suitability, we decided to postpone their analysis until we had reviewed the problem with the help of ampler data gathered from other countries.

*The Influence of Atmospheric Temperature and Humidity upon the Stages of Development of the Rat-flea (*X. cheopis*) and upon the Survival of unfed Adult Fleas.*

Periodic variation in the numbers of fleas infesting rodents is dependent upon climatic conditions. Bacot (1914), working in England with artificially regulated atmospheres, and the Plague Research Commission in Poona (1912), utilizing natural climatic variations, observed the effects produced by temperature and humidity on the phases of development of the rat-flea, and on the survival of unfed adult fleas. The interpretation of these observations is rendered difficult to the reader by the absence of grouping, and by the statement of the hygrometric records in terms of percentage humidity. We, therefore, converted the humidity data into the equivalent vapour pressure deficiencies (millibars), and grouped the individual observations in each set of experiments according to temperature and v.p.d.<sup>1</sup> The v.p.d. has the advantage over the relative humidity figure of giving a more exact measurement of the drying capacity of the air.

The method of grouping adopted by us shows that there is good agreement between the observations of Bacot and those conducted at Poona. Inevitable differences in the experimental methods affected principally the duration of life of unfed adult fleas, the conditions in Bacot's experiments having been more favourable for their survival, but the trend of the results in both sets of experiments is towards the same conclusions. We, too, made observations from January to June 1913 at Asyût on the period of survival of starved rat-fleas. The shortening of life, as the temperature and v.p.d. increased within certain limits, is plainly seen in Table III. The table shows also that the life of the Asyût fleas was prolonged during the hot months of June and July by adding moisture to the jars in which they were kept. Our analysis of the data as a whole leads us to conclude: (1) that for *X. cheopis* temperatures from 20° C. to 25° C. and v.p.d.s from 1 to 10 millibars are most suitable for its development at all stages; and (2) that the higher the temperature and the v.p.d.—a high deficiency indicating excessive dryness of the air—the shorter is the period of survival of adult fleas when they are kept unfed.

*The Influence of Atmospheric Temperature and Humidity upon the Seasonal Prevalence of Human Plague.*

We next tried to ascertain whether these conclusions fitted with epidemiological information relating to the seasonal prevalence of plague. We used first the data for six cities in India—Bombay, Poona, Belgaum, Lahore, Nagpur, and Rawalpindi—that were published by the Plague Research

<sup>1</sup> The symbol v.p.d. may be conveniently used to signify "vapour pressure deficiency" (saturation deficit).



Commission (1908). These cities were selected for study by the Commission, because they had suffered severely from plague for a number of years, and because they illustrated diverse climatic conditions. In order to eliminate irregularities in individual epidemics due to accidental causes, we thought it best to work out and tabulate for each city the mean or normal figures for human plague deaths, temperature, and v.p.d., grouped according to fortnightly periods and based on all the available years. When this was done, the figures for each period in the epidemic season were examined; the results did not agree well with our conclusions on the optimum conditions of temperature and humidity for the stages of development of the rat-flea; and the discrepancies were chiefly noticeable in the data for the up-country stations. However, the agreement progressively improved when the plague incidence for any fortnightly period in the epidemic season was compared, not with the corresponding temperature and v.p.d. figures, but with those of successive preceding fortnightly periods up to a limit of four to six weeks. And, further, when the results were plotted on charts, it was seen that the graphic correlation improved, for both temperature and v.p.d., by taking account of a lag period; this applied particularly to the data for Bombay.

Table III.

*Summary of observations showing the influence of climatic conditions upon the survival of unfed X. cheopis.*

SERIES A.						
Month	Mean temperature of month ° C.	Mean v.p.d. of month (mbs.)	Total fleas tested	Total days survival	Average days survival	Longest period of survival (days)
Jan.	11.6	3.9	418	1834	4.4	17
Feb.	12.6	5.5	1014	4442	4.4	16
March	15.1	7.1	1814	6970	3.8	12
April	23.1	13.8	1312	2791	2.1	6
May	25.2	17.8	1716	2829	1.6	6
June	26.3	19.6	1467	1906	1.3	3
SERIES B.						
(Moisture added to jars in which fleas were kept.)						
June	26.3	19.6	177	864	4.9	12
July	29.3	22.2	63	395	6.3	14

A study on these lines of other localities in India and of places outside India for which rat-flea counts were available strengthened our belief that the extent of the disease at any moment in the epidemic period is dependent upon atmospheric factors in operation some six weeks before, and not upon the strictly contemporaneous weather conditions. Moreover, since, even in places with diverse climates, the mean temperatures and v.p.d.s that prevailed six weeks before the height of the epidemic fall within a range varying from 20° C. to 25° C. and from 1 to 10 millibars, the conclusion seems to be justified that there is an optimum mean temperature and v.p.d. within these limits for the spread of the disease. The lag is reasonably explicable by the time taken for the complete development of *X. cheopis*.

The authors who have hitherto written on the subject have noted the wide range of temperature within which plague epidemics in different localities reach their maximum intensity, but they have not taken a lag period into consideration.

The graphic correlation for places, such as Lahore, where the diurnal temperature variations are extensive, is not so good as that for Bombay where the range of variation is smaller. We believe that the hourly temperature variations during the day deserve close attention in a study of the problem, because they must exert a constantly changing influence either in the direction of stimulating or curbing flea development. A useful inquiry would be to compare the mean diurnal temperature variations and the human plague data for corresponding weekly or fortnightly periods, with special reference to the lag interval which we have defined. If it were possible to procure the necessary figures—up to the present we have not succeeded in doing so—the number of favourable hours, on the assumption that there is an optimum range from 20° C. to 25° C., could be extracted and compared with the human plague data. We have attempted an approximate analysis for Lahore, and found that the results conformed to the views expressed above.

*The Relative Influence upon the Seasonal Prevalence of Human Plague of the Temperature of the Air and of its Dryness as measured by the Vapour Pressure Deficiency.*

Maritime cities such as Bombay, Hong Kong, Madras City, Colombo, Surabaya (in Java), Sydney, and Marseilles, possess a humid climate; the v.p.d. is, as a rule, less than 10 millibars. We conclude that in those cities the temperature of the air is the chief factor accounting for the varying flea-rate and the prevalence of rat and human plague. The Plague Research Commission in their later work in India attributed primary importance to the factor of relative humidity in checking plague epidemics in the hotter and drier up-country stations, but we think that the influence of the temperature in these places cannot be ignored. In localities where extreme drought prevails during the fall of the epidemic the influence of the v.p.d. is difficult to estimate, for the reasons that this factor has a temperature component, and that the temperature and v.p.d. usually run parallel. In some places, however, a v.p.d. above 10 millibars is able to restrain the spread of plague when the temperature is favourable; and Poona may be mentioned as a case in point.

*The Relation of Departures either in Excess or in Defect of the Optimum Range of Temperature to the Extent of Spread of the Disease in Different Localities.*

The degree in which the climate of an infected locality approximates to the optimum is apparently a material factor in determining the normal mildness or severity of plague within it. In maritime cities, where the v.p.d. is low, the prevailing temperature throughout the year may be such as to inhibit the disease. Colombo, with many opportunities for importing the infection, is an example; it has never been badly infected. The mean monthly temperatures are consistently higher than those of Bombay and vary from 26.1° C. to 28.7° C.; the flea average is low. Surabaya, a seaport, is relatively immune from plague, as compared with other localities in Java. The climatic conditions of this town are very like those of Colombo, the mean temperatures varying from 26.0° C. to 28.1° C.; in Surabaya, too, the flea average is low.

*The Analysis of the Epidemic Curve throughout the Year.*

There are two types of epidemic curve for human plague. The normal figures for Bombay, Calcutta, and Hong Kong give symmetrical curves; in Lahore, Nagpur, Cawnpore, and Lucknow the fall is more rapid than the rise, and the curves are therefore asymmetrical.

*Symmetrical Epidemic Curves.* In Bombay not only is the epidemic curve symmetrical, but also that of the epizootic (for 1906) and of the flea-variations (for 1907). The portion of the curves representing the epizootic and epidemic periods is explained by the rat-flea variations, which are mainly a function of temperature, since the v.p.d. is rarely unsuitable. The non-epizootic and non-epidemic seasons are accounted for by the high temperatures then prevalent, which diminish the numbers of fleas owing to their unfavourable action on the developmental stages, and which shorten the life of infected fleas whose valve of the proventriculus is inoperative.

*Asymmetrical Epidemic Curves: Lahore, Nagpur, Cawnpore, and Lucknow.* It is unfortunate that for these cities no epizootic curves are available for comparison, but probably they too would prove to be asymmetrical. We have not met with an asymmetrical flea-curve from a tropical locality; those of Cawnpore, Lucknow, and Asyût, in part associated with high temperatures and v.p.d.s, are symmetrical. The flea-variations are again the principal factor in this class of epidemic curve, but an additional modifying circumstance is present, and we suggest that this may be found to reside in the wide range of diurnal temperature variations appertaining to those localities. We surmise that the temperature of some part of the day rises so far above the optimum as to exert a retarding influence upon the transference of the infection to rats and human beings, by shortening the life of infected fleas in which the proventricular valve is imperfect.

In Bombay, where the amplitude of the diurnal temperature wave is smaller than in the up-country cities, excessive temperatures are also lethal to infected fleas, but the effect begins to appear only towards the close of the epidemic period; in Lahore, on the other hand, the influence of high temperatures appears earlier, namely, at the time of maximum plague prevalence. The difference, then, in the character of the two classes of curve depends upon the time in the course of the epidemic when high temperatures begin to operate effectively. The element, whether temperature, or, as some may think, v.p.d., or a combination of these, that distorts the normal epidemic curve, produces its effect within a brief space of time, for it acts directly on the insect carrier of the infection.

The process of thinning out that goes on in the plots of infection where rat plague exists, as the result of the death of infective fleas within them, may be conceived as operating continuously and in uniformly varying degrees throughout both types of epidemic. This hypothesis implies a superposition of variation curves, one of them referable to the breeding of fleas, and the other to the

infected part of the flea population; the effects of abnormally high temperatures are seen, however, only during the fall of epidemics of the asymmetrical type. Analysis of the diurnal temperature variations will lead, we think, to a promising line of attack upon this aspect of the problem.

Temperatures below  $20^{\circ}\text{C}$ . hinder the multiplication of fleas, and accordingly also check plague. In Lahore, the months that immediately precede the epidemic season are characterized by low mean temperatures:  $11.2^{\circ}\text{C}$ . is the lowest normal mean monthly temperature; whereas the non-plague months following the epidemic are marked by high mean temperatures ( $25^{\circ}\text{C}$ . to  $35^{\circ}\text{C}$ .). The curves of Tongshan in Manchuria are probably typical of localities with a temperate climate. Here, an increase in the number of fleas runs parallel with temperatures rising to the optimum zone, a decrease in their numbers with temperatures falling to zero.

*The Relation of Climate to the Seasonal Prevalence of Plague in Egypt.*

Reverting now to the Egyptian data, we find that the normal temperatures and v.p.d.s for the month preceding that of maximum plague prevalence in the four divisions of the country are, from south to north, respectively:  $14.9^{\circ}\text{C}$ .,  $20.5^{\circ}\text{C}$ .,  $22.1^{\circ}\text{C}$ .,  $23.8^{\circ}\text{C}$ ., and 7.9, 11.7, 9.8, and 7.9 millibars. These figures are in good agreement with the views we have expressed for optimum plague conditions. We reach the conclusion that the delay in the diffusion of plague in Egypt from south to north is correlated with the climatic variations throughout the country.

*Conclusions.*

We are conscious that it is not easy to estimate the influence of the climatic factors implicated in the seasonal prevalence of bubonic plague. With due reservations we would summarize our conclusions as follows:

(1) The extent of the disease at any moment in the epidemic period is determined not by the strictly contemporaneous weather conditions, but by those which existed four to six weeks previously. The interval is attributable to the effect of atmospheric conditions on the various stages of development of the rat-flea (*X. cheopis*).

(2) When this lag period is taken into account, there is found to be an optimum range of temperature from  $20^{\circ}\text{C}$ . to  $25^{\circ}\text{C}$ . and of vapour pressure deficiency from 1 to 10 millibars for plague prevalence; these, too, are the optimum limits for flea development.

(3) The degree in which the climate of a locality approximates to the optimum determines to a definite extent the normal character, whether mild or severe, of its plague history.

(4) In maritime localities the atmospheric temperature is a more important factor in regulating the prevalence of the disease than the vapour pressure deficiency. In places with a hot dry climate the rise of the epidemic is correlated with the temperature variations; a high prevailing temperature or excessive dryness of the air (vapour pressure deficiency) or a combination of these hastens its decline by increasing the mortality in the infected part of the rat-flea population, and thus restricting the transference of the infection.

In certain localities, when the temperature is favourable, a dry atmosphere with a vapour pressure deficiency above the optimum limit is able by itself to check the spread of the disease.

(5) In the dissection of the climatic factors associated with plague, the diurnal temperature variations deserve attention.

(6) The gradually increasing delay in the onset of epidemic plague from south to north of Egypt is correlated with the seasonal variations in the climate throughout the country. The analysis leading to this conclusion indicates that, in agreement with the data from India and elsewhere, the seasonal prevalence of human plague in Egypt, as deduced from the massed statistics, is dependent upon the factors governing the prevalence of rat plague, and, in particular, the climatic factors that influence the life history of the rat-flea (*X. cheopis*).

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GENERAL CONSIDERATIONS ON THE EPIDEMIOLOGY OF  
BUBONIC PLAGUE IN EGYPT.

Plague as observed at the present day in Egypt does not equal in local intensity and widespread diffusibility the older epidemics that overran the country, or the epidemics of recent years in India. The number of cases that were notified in Egypt from 1899 to 1919 inclusive, namely 14,783, has been exceeded in several single years in the city of Bombay alone. This relative exemption does not seem to be due to a scarcity of rats and fleas, the cardinal requisites for epidemic spread; but the Egyptian rat may have a higher average immunity than the Indian rat, inherited from the survivors of the continually recurring epizootics of the seventeenth and eighteenth centuries. Thus plague was scarcely ever absent from Egypt during the period of 243 years dating from the commencement of the seventeenth century; the records we have consulted speak of its presence in nineteen out of the twenty-four decades of this period. The prolonged succession of plague-stricken years was followed by a free interval of fifty-five years: from 1844 until 1899. In India, on the other hand, the two generalized epidemics prior to the year 1896 belong to the seventeenth century: one in 1616 in the time of the Emperor Jehangir, of which it is said that it devastated the country for eight years, and the other in 1688 which lasted for seven or eight years in the reign of Aurungzeb (Creighton).

In November 1913 we made observations on rodents trapped from houses in two native quarters in Cairo, namely, the Musky, the bazaar quarter in the centre of the city, and Bulâq, which is adjacent to the Nile and opposite the island of Gezîra. 5400 traps were set; they caught only 88 *R. rattus* (equal to 1.6 per cent. traps set and 176 per cent. pure takes) and 129 *Acomys* (equal to 2.4 per cent. traps set and 141 per cent. pure takes); seven weasels (*Mustela africana*) and one mouse were also trapped. These results show that the rodent infestation of native houses in Cairo is very small, and they explain the immunity of the city to plague. The fewness of rats is probably associated with the substantial construction in stone of the buildings and with the commonness of weasels in the native houses.

The distribution of the plague cases in the Abnûb and Qûs outbreaks indicates that the spread of infection in these towns was haphazard. If it were possible to ensure that the human agent remained wholly passive, the infection would proceed in a regular manner outwards from a centre, the first focus. The best evidence for this belief that we have found is the experiment related by the Indian Plague Research Commission (1907), in which the human population of an infected and evacuated village near Bombay was represented by guinea-pigs. Here the uniform progression of the epidemic amongst the experimental animals from the first infected house proves indirectly that the excursions of the rats (*R. rattus*), while a severe epizootic was going on, were of short range; our own evidence on the movements of

the Egyptian *R. rattus* points in the same direction. It is true that some of the scattered nuclei in local outbreaks of rat and human plague may be due to fresh importation of infection from outside the locality, but we think that internal rather than external causes are at work in their formation. We believe that the people themselves are largely responsible for the spread of the infection by their movements to and fro and by the removal to distant parts of the town of relatives who are ill or of bundles of clothing or other articles. And, further, we suspect that measures for disinfection are a means of spreading the disease, not only by leading to concealment of plague patients in an endeavour to evade sanitary interference, but also through the agency of the disinfecting staff.

It is naturally difficult to ascertain by actual observation precisely how the infection is conveyed from one part of a town to another; our experience agrees best with the conclusion arrived at in India that it is conveyed in the rat-flea.

It is an axiom that the spread of plague in a country is directly related to its means of communication, whether by rail, road, or river; and that the scattering of infection is hindered or favoured by the simplicity or complexity of the network.

Egypt has an excellent system of railways, with a total length in 1912 of 1481 miles; in 1911 nearly 28 million and in 1912 nearly 29 million passengers travelled by rail in Egypt. The records give instances of the detection of persons suffering from plague while travelling by train. It has been already shown that the native river-craft ("feluccas") which voyage along the Nile from Aswân town to its mouths at Rosetta and Damietta are rat-ridden. Highways and agricultural roads hardly exist, but there is an immense network of field paths and riding tracks between the towns and villages. The river in its course through the cultivated areas is not a serious barrier to the passage of the infection; the barrages across it serve as bridges; and there are eighty-nine Government ferries in Upper Egypt. Feluccas are means of transit across the Nile at any part of its course.

Markets (agricultural produce and cotton) increase the movements of the people during the epidemic period, and so help to spread the disease. From information supplied to us we learn that 180 *mulids* (fairs of a semi-religious character lasting from one to four weeks) held annually over the whole of Egypt attract at least three-quarters of a million visitors and last altogether for 1300 days.

A scheme was organized in 1913 whereby the clothes of plague patients could be sent to our laboratory at Asyût from towns or villages in other provinces in order that they might be examined for rat-fleas and for infectivity to guinea-pigs. Specially designed flea-proof bags were used for the conveyance of the clothes. On arrival at the laboratory the bag and its contents were transferred to a roomy flea-proof sack, held open by bamboo stakes, into which a guinea-pig was put and kept for at least ten days. The year 1913 was an exceptionally mild plague year, and the seventeen experiments that we made—the results were negative in all—were not convincing, because we were unable to obtain in each case satisfactory evidence of the exact nature of the patient's illness. The clothes were sent from patients who were suspected of having contracted plague, but the bacteriological diagnosis was reported as positive from only one of them. We prefer to trust to observations in plague

localities that strongly impressed us with the likelihood of infective rat-fleas being conveyed from place to place and so starting fresh epizootics. The plan that we adopted might, however, prove in more advantageous circumstances a valuable means of estimating the risks of infected fleas being carried in clothing.

Our observations at Asyût on the duration of life of unfed rat-fleas (Table III) showed that, as the weather in the plague season became hotter and drier, their length of life diminished; and we conclude that under natural conditions the diffusion of plague receives a corresponding check.

The rôle of the human flea as a possible plague carrier was investigated by the Plague Research Commission in India (1907). They thought it unlikely that this flea conveys the infection from man to man, because the septicaemia in human beings as compared with that in rodents is so slight that the chance of the flea becoming infected is negligible. We made a number of observations at Asyût on the infestation by human fleas of clothes taken from unselected patients who had been admitted to the adjoining Government Hospital but were not suffering from plague (Tables IV and V). The results are interesting apart from the question of the ability of the human flea to convey plague infection. They indicate that the numbers are greatest in February, March, and April, and that they diminish as the weather becomes hotter. Fleas (*P. irritans*) and lice were present in 89 per cent. of the lots of clothing. The number of fleas was sometimes startling; thus, from the rather superior clothes of an old man, more than 1000 fleas and innumerable lice were removed, as well as a considerable number of bugs (between forty and fifty) and a variety of other insects; 538 of the fleas were examined, and all of them were found to be *P. irritans*.

Table IV.

*Showing average number of human fleas in clothes of plague-free hospital patients at different seasons of the year. (Asyût town, 1913.)*

Period	No. of patients	Fleas	Average per patient
Feb., March, April	127	4002	31.5
May, June, July	58	382	6.6
Aug., Oct., Nov.	28	52	1.9
Totals:	213	4436	20.8

Table V.

*Showing distribution of human fleas and lice in clothes of plague-free hospital patients at different seasons of the year. (Asyût town, 1913.)*

Period	No. of lots of clothing	Fleas, no. of lots of clothing with							Lice, no.	
		0	1-5	6-20	21-50	51-100	101-200	201 and over	infested with lice	no lice
Feb., March, April	128	1	15	56	31	17	6	2	113	15
May, June, July	58	10	28	15	5	0	0	0	55	3
Aug., Oct., Nov.	28	13	13	2	0	0	0	0	22	6
Totals:	214	24	56	73	36	17	6	2	190	24

Bubonic plague may become extinct without any obvious cause in a country where it has been rife; for example, it disappeared from Egypt in the year 1844 and from India in the seventeenth century. The chief reasons for its virtual extinction in Europe are, first, the supplanting of *R. rattus*, the house rat, by *R. norvegicus*, the outdoor rat; and secondly, the improved housing conditions, which have accompanied the general advance in the



standard of living. The diversion of trade routes, by lessening the chances of reimporting the virus, has helped in the past to keep the disease within bounds; thus the decrease in the intercoastal trade between the countries bordering on the Eastern Mediterranean which is said to have taken place near the middle of the nineteenth century has been assigned as one of the reasons for the cessation of plague at that time in Egypt. The fundamental causes are, however, doubtless traceable to the rat and the rat-flea. It is impossible with our present knowledge to predict the future course of the disease in a country like Egypt where the conditions seem to be favourable for its continuance; nor can we even explain satisfactorily the fluctuations in its prevalence from year to year. But since there are only a few references in the literature to the behaviour of plague during epidemic cycles, we may note briefly some unfamiliar aspects of the subject.

A long succession of plague epidemics in a city or district has the effect of increasing the resistance of the rat population, not only by eliminating susceptible individuals but by the transmission of immunity to the progeny of the survivors. (Plague Research Commission, 1912.) This factor operates slowly and, if it were the sole cause, we should expect it to lead to a gradual disappearance of the disease.

The relative abundance of the fleas infesting rats has also to be considered. A diminution in their number below a critical point, if it were maintained during the plague season, would soon result in the infection either ceasing to spread or spreading at a slow rate. Our observations in Asyût on the prevalence of rat-fleas are significant here. The monthly averages for *R. rattus* and *Acomys cahirinus* were uniformly higher throughout the year 1912 than in the following year; the incidence of human plague, too, was greater in 1912, for there were 687 cases in this year in Upper Egypt as compared with 335 cases in 1913. The weather, as we have seen, has an influence upon the breeding of rat-fleas, but the climatic variations during those two years do not appear to us to be such as to account wholly for the differences in the flea prevalence; and it is quite conceivable that other factors were at work.

We devoted attention to the natural enemies of rat-fleas and their larvae, and collected various insects from houses at Kôm Ombo and Asyût, which we submitted to Mr Bacot for identification and for his opinion regarding their ability to act as a check to the breeding of fleas. He thought that the larval stage of the flea is the one at which such a check is brought about, for the flea larvae are subject to attack by other insects and their larvae; and that among the insects in our collections certain small beetles belonging to the group "Staphylinidae" and minute yellowish ants of the group "Leptothorax" are likely to be of importance. It had occurred to us that the exceptionally heavy rat-flea infestation at Kôm Ombo might have been in part associated with the recent foundation of this estate; we supposed that there had not been enough time for insect enemies of the fleas to become established in the nests and burrows of the rats. Mr Bacot, to whom we referred the point, wrote to us: "Your suggestion is very probably an explanation of the flea prevalence, and fits in with the known facts concerning insects in a new environment."

There are scattered publications which deal with the parasitic invasion of rat-fleas by bacteria, protozoa and helminths, but systematic observations have not yet been made in countries where plague is endemic with a view to

discover whether internal parasites adversely influence the flea population in any important degree. Investigations in plague-infected districts on the enemies and parasites of the rat-flea are desirable, and might help to make clear unaccountable fluctuations in the prevalence of bubonic plague from year to year, both locally and over extensive tracts of country, as well as the causes that lie at the root of the natural decline and termination of a recurring series of epidemics.

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#### THE EPIDEMIOLOGY OF PNEUMONIC PLAGUE IN EGYPT.

When we survey the cumulative experience of the great epidemics of pneumonic plague in recent years throughout the world, certain features are seen to be common to all. First, the disease prevailed amongst peoples who were entirely ignorant of the means of self-protection, and who were unassisted by any sanitary organization capable of arresting its course. Secondly, the housing conditions were characterized by gross overcrowding and by the absence of any provision for ventilation. The inferences may be drawn that popular ignorance, administrative deficiencies, overcrowding, and bad ventilation are the essential factors that contribute to the spread of pneumonic plague; and that the extent of spread in an outbreak, in whatever country it may occur, is determined by its particular set of co-operating factors and by the degree in which each is present.

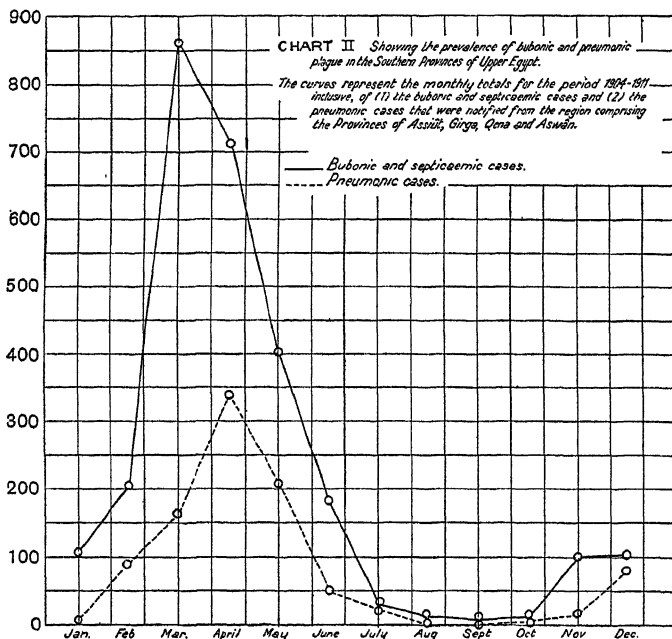
The incidence of pneumonic plague has been fortunately low—1 to 5 per cent.—in localities that have suffered severely from bubonic plague during the present pandemic, for example India, Hong Kong, and Java. In contrast with these localities Upper Egypt—particularly its southern provinces, Qena and Girga—manifests a decided inclination to kindle pneumonic plague foci. In the period from 1899 to 1913 there were altogether eighty-eight pure outbreaks of pneumonic plague in Egypt. Viewing the outbreaks as a whole we find that the number of cases in each is small, and that the majority remained isolated. When the escape of contacts from the primary outbreak led to the introduction of the disease into neighbouring villages, there were seldom more than three or four secondary offshoots. It is clear then that, although in the affected districts foci are apt to flare up, the outbreaks that follow are characterized by low diffusibility.

In Egypt the ignorance of the people and their habit of clinging to traditional customs and prejudices encourage direct transference of the infection. The practice of crowding round and kissing the sick, the assemblage of men and women at funeral ceremonies, and the attempts that are made to conceal deaths and to evade the measures for controlling the disease, add greatly to the difficulties of the Sanitary Administration. Fortunately not all the main elements of spread are present, and this accounts for the low diffusibility.

Although overcrowding takes place on special occasions in the manner indicated, it is not a normal feature of Egyptian domestic life. Further, owing to the hot weather in Qena and Girga during the pneumonic plague season the people keep their houses sufficiently well ventilated, and, moreover, live a good deal in the open air. Again, the restricted regional distribution of pneumonic plague in Egypt makes it possible to strengthen the defences against its spread. The smallness of the individual foci, as contrasted with the extensive epidemics within recent years in China, is not due to any enfeeblement of contagious quality; clinical and bacteriological evidence revealed no difference in the character of the disease from that observed elsewhere; the cases without exception were fatal. We attribute the small scale of the outbreaks to the alertness and active intervention of the Sanitary authorities, to ample ventilation of the houses, and to the absence under normal conditions of domestic overcrowding.

#### *Seasonal Prevalence.*

Chart II represents the monthly totals for the period 1904-11 inclusive, of (1) the bubonic and septicaemic cases, and (2) the primary pneumonic cases,



whether from pure or mixed outbreaks, that were notified from the region comprising the provinces of Asyût, Girga, Qena, and Aswân. The close correspondence between the epidemic curves makes it practically certain that pneumonic plague in this region originates locally from bubonic or septicaemic plague. We are indebted to Mr J. I. Craig for working out the coefficient of correlation of the figures from which the curves were plotted;  $r = +0.857$  for bubonic and septicaemic cases correlated with pneumonic cases of the same month,  $+0.880$  with one month's lag, and  $+0.291$  with two months' lag: the best fit was obtained for a lag varying between a fortnight and a month<sup>1</sup>. Although it can scarcely be doubted that the majority of the pneu-

<sup>1</sup>  $r$  lies between 0 and  $\pm 1$ ,  $\pm 1$  indicating complete association.

monic cases arise locally, and prevail within fairly narrow limits contemporaneously with bubonic cases, pure outbreaks in the southern provinces sometimes originate in the local non-plague season. We had experience of three of these off-season outbreaks, namely, at Khattara (Qena Province), Zarâbi (Girga Province), and Damanhûr (Asyût Province). Of eighty-eight pure outbreaks in Egypt during the years 1904-13 inclusive 76 fell within the local plague season and the remaining twelve within the local non-plague season; all of the latter group broke out in villages in the southern provinces of Upper Egypt, and 72 out of 76 in the former group attacked the provinces of Qena, Girga, and Asyût. This analysis includes sixteen instances in which only one case of pneumonic plague was found; but we have judged it right to regard them as potential outbreaks, for we believe that those cases remained single on account of the vigilance of the Public Health staff. The off-season outbreaks are derived from patients suffering from bubonic plague who travel from the Delta when the disease is prevalent there. A good deal of the casual labour of the Delta is undertaken by temporary migrants—mostly males—from the southern provinces of Upper Egypt, especially Qena and Girga; and it is known that, when attacked by illness, they seek to return at once to their homes. The high mortality amongst the plague-stricken travellers is associated with the strain of the journey, and is probably often due to a septicaemia or a secondary plague pneumonia.

#### *Regional Distribution.*

The heavy incidence of pneumonic plague upon the provinces of Qena and Girga and the lessening incidence towards the north are difficult to explain. In order to compare the climatic conditions in localities which have suffered from pneumonic plague, we obtained the data for temperature and vapour pressure deficiency relating to the epidemic periods in Southern Upper Egypt, Johannesburg, Srinagar (Kashmir), and Changchun and Mukden (Manchuria). The figures show that the weather in Qena and Girga during the prevalence of pneumonic plague is exceptionally hot and dry. This result does not fit in with the view of Teague and Barber that the extent of spread of the Chinese epidemics is associated with the low water deficit of the air due to the cold weather when these outbreaks prevailed; they think that the moist atmosphere protects the bacilli in the droplets of sputum from desiccation. The Egyptian experience confirms our belief that the primary factors concerned in the widespread dissemination of pneumonic plague in countries where the disease spreads during cold weather are those we have already mentioned, and that the influence of atmospheric conditions upon infected sputum particles is less important.

We sought for any significant circumstances peculiar to the Qena-Girga district. The southern part of Upper Egypt from Asyût to Aswân is the only region in the country where perennial irrigation does not exist, so that there is no summer cultivation and the ground during the plague season is dry and parched. It has occurred to us that by increasing local

drought the lack of irrigation and cultivation during the plague season in Qena and Girga may be a factor predisposing to the onset of a secondary pneumonia in bubonic and septicaemic cases. Excessive evaporation of moisture from the pulmonary mucous membrane due to a high saturation deficit of the air may conduce to the production of patches of secondary pneumonia; this is more likely to occur if the mucosa is congested as in those plague patients in whom there is a blood infection.

We looked, too, for indications of varying susceptibility to plague infection amongst the Egyptian natives. Direct evidence was not obtainable from the plague statistics, but evidence of ethnological variations was found in Craig's study of the anthropometry of modern Egyptians. The Qena-Girga district forms from this point of view a well-defined unit, and indeed the racial grouping worked out by Craig corresponds fairly well with the distribution of pneumonic plague over the country. It may be that the ethnological differences connote variations in susceptibility to pulmonary plague infection.

Table VI.

*Showing the regional distribution of pneumonic plague in the provinces of Egypt; the figures represent the total notified cases of each type for each locality summed for the period 1904-12 inclusive.*

Region	Bubonic and septicaemic cases	Pneumonic cases	Percentage of pneumonic to total cases
Aswân province	535	7	1.3
Qena province	1192	586	33.0
Girga province	312	218	41.1
Asyût province	1071	150	12.3
Minia province	964	62	6.0
Beni Suef province	401	13	3.1
Fayûm province	502	44	8.1
Giza province	18	—	—
Delta provinces	1603	58	3.5
Suez and Ismailia	98	11	10.1
Alexandria, Damietta and Port Said	912	24	2.5

*The Clinical Forms of Pneumonic Plague and their Epidemiological Relations.*

The two clinical forms are: (1) primary pneumonic plague arising from direct infection of the respiratory tract, and (2) secondary plague pneumonia coming on as a complication in a bubonic case or in one without obvious buboes (septicaemic plague); whether buboes are present or not a blood infection may be assumed. If exceptional cases such as laboratory infections are left out of account the primary form of the disease, in whatever country it is found, may be traced to a plague patient with a secondary pneumonia.

In endemic centres a rapidly and invariably fatal disease attacking several members of a family should at once suggest a diagnosis of primary pneumonic plague; but in Egypt confusion may arise from the concurrence of typhus fever, which is common in the plague season. Here, typhus fever is often difficult to diagnose clinically, for in general it runs a mild course and the rash may not be characteristic. In Upper Egypt the season for relapsing fever is also coincident with plague, but the differential diagnosis is much easier and can be confirmed by examination of the blood. A simple, rapid, and certain means of post-mortem diagnosis consists in the preparation of smears of material taken with a needle and syringe from the apex and base of each lung and from the heart. In our notes of lung and heart-

blood smears from twenty-seven cases of pneumonic plague the bacilli are stated to be on the average most numerous in smears from the right apex, diminishing in the order: the right base, the left apex, the left base, and the heart-blood. The preparations often resembled those made from a pure culture. The conditions under which we worked were not suitable for withdrawing material from the lungs during life, but we sometimes used this method, and we believe that it will prove to be a valuable diagnostic aid where it can be conveniently employed.

We were able to obtain evidence that a secondary pneumonia is apt to supervene in persons who travel while they are suffering from plague. Apart from a natural desire to return home when they fall ill, these patients may be urged on by the impulse to wander, which is one of the symptoms of plague; some are able to get about fairly well, even when the disease is far advanced. The liability of ambulant plague patients to a secondary pneumonia may be explained as follows. The walls of the blood-vessels of the bubo tend to rupture as the result of softening brought about by the inflammatory process. Infective thrombi form in the vessels; and if they become detached the bacilli may be swept into the circulation and may reach the lungs. Muscular efforts made by the patient must greatly increase the risk of a pulmonary complication.

Gotschlich (1899) affirmed that virulent plague bacilli can be found in the sputum obtained from patients in the convalescent stage of a plague pneumonia, even after several weeks have elapsed from the onset of the disease. This observation, which was based on the examination of one case of primary and two cases of secondary pneumonia, has been often cited, but, so far as we are aware, it remains uncorroborated. He stated that the plague bacillus was isolated by inoculating guinea-pigs intraperitoneally with the sputum, but no mention is made of any tests used for identifying the plague-like bacteria that were recovered. We have never met with such a carrier, nor with a pneumonic plague patient who was thus infected. Moreover, we have been unable to find in the Egyptian records any suggestion of such a mode of origin of pneumonic plague. When evidence on this important question is brought forward, the details that are given should be as full and unequivocal as possible.

### *Types of Outbreak.*

In Egypt, two types occur: (1) mixed outbreaks, and (2) pure pneumonic foci. The former is the normal type in the provinces of Qena and Girga. Thus, in a mixed outbreak, previously described, of eighty-two cases of all forms at Qûs town, there were fifteen cases of primary pneumonic plague, which were distributed in five groups, each with a different originator. Pure outbreaks originate either from a primary pneumonic case or from a bubonic patient with a secondary plague pneumonia who has come from another locality. They are not accompanied by any bubonic plague, and are not directly related to rat infection.

A guinea-pig was kept for three days in each of eight houses of patients in Edfa village, and was examined daily for fleas. On five of the guinea-pigs no fleas were caught, and the total counts on the others were 2, 8, and 16; all the animals remained healthy. The fewness of the rat-fleas contrasts with the results obtained elsewhere in houses in which there were plague-infected rats.

### *Inter-local Movements of the People that promote the Dispersion of the Disease.*

Temporary migrations associated with local economic and labour conditions are particularly noticeable in the provinces of Qena and Girga, and are due to the gap in the succession of the agricultural seasons caused by the partial

irrigation system. The introduction of the system of perennial irrigation into the southern provinces may be expected to act indirectly as an effective plague measure by increasing their fertility and so stabilizing the population.

*General Conclusions on the Origin, Spread, and Decline of Pneumonic Plague.*

(1) Outbreaks of pneumonic plague take their immediate origin from patients with bubonic or septicaemic plague in whom a secondary pneumonia has supervened; they may assume the form of scattered foci in localities in which a bubonic epidemic is proceeding (mixed outbreaks); or they may occur as pure epidemics.

(2) Ignorance of the means of self-protection, deficiencies in the Sanitary Administration, overcrowding, and the absence of proper ventilation are the primary circumstances that encourage the spread of the disease.

(3) The decline and cessation of epidemics are associated with a return to healthier conditions of life, in particular, a free circulation of air within the houses and a diminution of overcrowding.

(4) The range of contagion in pneumonic plague is so close that direct transference of the infection must often occur. Mediate infection from a high concentration of sputum particles in the air doubtless happens when the ventilation is inadequate. The influence of atmospheric conditions upon infected sputum droplets is of secondary importance. An essential requisite for its spread is the close contact between the sick and the healthy that results from overcrowding or from the habits of those exposed to risk.

(5) The prevention of pneumonic plague in man depends in the last resort upon the extinction of plague in rodents.

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#### THE PREVENTION OF PLAGUE IN EGYPT.

The subjects usually discussed under the head of plague prevention are: (1) the exclusion of rats from houses; (2) the reduction in numbers or the extermination of rats infesting houses; (3) the removal of the infection within buildings when plague has appeared; and (4) the prophylactic use of a plague vaccine. In principle, these measures need no defence, but it is necessary to inquire whether the success to be expected under the most favourable conditions may not be modified in practice by circumstances peculiar to the country in which it is proposed to apply them.

The work at Asyût during two years showed that persistent trapping had the effect of appreciably reducing the numbers of rats infesting the houses. The reduction was due solely to trapping and not to any discoverable epizootic disease; the rats remained free from plague throughout the period of observation. Four small villages were trapped as thoroughly as possible by setting traps daily in the houses for several weeks. The results are similar in all, and indicate a near approach to extinction of the rodent population. They suggest that the Egyptian people themselves could virtually abolish the risk from plague if, instead of a surprising tolerance, they were possessed by such a dislike for rats as would call forth persistent efforts to exterminate them.

In theory plague is one of the easiest as it is also in practice one of the most difficult diseases to combat. The communities of Western Europe have successfully solved the problem—not of set design but in consequence of the gradual progress, both social and material, during the last two centuries. The risk of a widespread epidemic of bubonic plague in these countries is, we believe, negligible, and even if plague should become epizootic amongst the field rats, a considerable epidemic of human plague is unlikely to break out, so long as the modes and standards of living do not change for the worse. An adequate idea of the distance which must be travelled before Egypt enjoys a similar security can be got only by observation at close quarters in the



country. Improvement in the housing conditions is desirable, but is scarcely feasible on a large scale. The ignorance of the mass of the people, confirmed by age-long prejudices, effectually hinders them from understanding and willingly submitting to logical methods of dealing with epidemic diseases. In our judgment the outlook may be indicated in the statement that progress in plague prevention will be found to keep pace with the course of national development.

# THE INFLUENCE OF WEATHER CONDITIONS ON THE MORTALITY FROM BRONCHITIS AND PNEUMONIA IN CHILDREN.

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(With 3 Diagrams.)

THE mortality from the two diseases of the respiratory system, bronchitis and pneumonia, in children is commonly accepted to be largely dependent on the incidence of unfavourable weather conditions which predispose to infection, but there is not unanimity of opinion as to the meteorological factors which are most closely associated with their onset.

The following paper is based on the results of an investigation which was undertaken to try to determine whether any further information on this subject might be obtained by the method of correlation. The data chiefly utilised were obtained from the four Scottish cities, Glasgow, Edinburgh, Aberdeen and Dundee, and from London, and consist (1) of the monthly totals of the deaths of children under five years of age from bronchitis and pneumonia, respectively, in each city; (2) of the weekly totals of the deaths of children in London from the respective diseases; and (3) of the monthly totals of deaths from respiratory diseases, or bronchitis and pneumonia summed together, in children under five years in each of the four Scottish cities; and of the weekly totals of deaths from the two diseases combined in London children. For Glasgow, Edinburgh and Aberdeen, figures were taken for a period of 54 years, 1857–1910; for Dundee for 45 years, 1866–1910; and for London for 44 years, 1876–1919. The numbers of deaths in each month for the several cities as well as in each week for London were extracted from the annual reports of the Registrars-General for Scotland and England and, before use, were corrected for the increase of the population under five years that took place during the period of years under review. The meteorological factors, which were recorded for the cities for the same period of years and with which the totals of these fatal cases were correlated, were also extracted from the annual reports of the Registrars-General and comprised:

1. Temperature:
  - (a) the monthly and weekly mean temperature;
  - (b) the monthly and weekly mean minimum temperature.
2. The monthly and weekly mean relative humidity.
3. The monthly and weekly mean rainfall.

In addition to these data for the five cities, the death-rates from bronchitis, pneumonia and respiratory diseases, respectively, in children under five years of age in the 55 registration counties of England and Wales for the decennium, 1901-1910; and the death-rates from respiratory diseases in the same age group in the 42 registration counties of England in the decennium, 1891-1900, were also extracted from the decennial reports and were correlated with the mean annual temperature and mean annual rainfall for the corresponding counties. These meteorological factors for the counties were determined in a special manner to be described later.

The present investigation in regard to the two diseases was restricted to their incidence in children in the age period 0-5 years, because this group is well defined, has a high mortality, and comprises individuals, who, in the great majority of instances develop a similar type of pneumonia, viz., broncho-pneumonia, are probably, in large measure, similarly susceptible and similarly exposed to the influence of the weather in the different cities; and on whom the effect of other adverse agencies may be regarded as more or less equal. The recorded mortality from respiratory diseases in children, moreover, while influenced doubtless in some degree in the more recent years by the fashion of recording measles, whooping cough, etc., the primary cause, as the cause of death instead of bronchitis or pneumonia, the terminal cause, as may possibly have been done not infrequently in earlier years, has not, like that for adults, been markedly modified during the last three decades by epidemics of influenza. The data are thus more truly comparable than would be those of adults for the longer period of years that has been considered. It is intended, however, to issue later a separate study of the relationship of weather conditions to the mortality from respiratory diseases in people at the other extreme of life for the shorter period of years up to 1890 about which time influenza became epidemic.

A survey of the data suggests that there has been a transference of cases from bronchitis to pneumonia in recent years in accordance with change of fashion in diagnosis. This is a strong argument in favour of combining the figures for the two diseases for the purposes of the investigation. It has been decided, however, to examine the mortality data for each disease separately, as well as for the two combined, in the cities to see what differences may be elicited as it is not certain that they are influenced exactly in the same way by weather conditions.

With regard to the data utilised, it must be conceded that the totals of fatal cases may not be a very accurate index of the number of cases that actually occurred as the mortality in both bronchitis and pneumonia is variable and need not run exactly parallel to the incidence of the two diseases. It must also be acknowledged that some of the cases recorded as terminating fatally in any one month, may not, owing to the variable duration of the respective diseases, have originated in that month. By taking the aggregate deaths for such a long period as a month, however, it was hoped that the great majority

of the cases ultimately fatal would be correlated with the mean values of the weather factors coincident with the onset of the diseases therein, although great accuracy in this respect could not be expected. For the Scottish towns, with the possible exception of Glasgow, it was practically essential to use the monthly figures as the data for the weekly deaths were not sufficiently numerous for the purpose of correlation. In the case of London, however, where the numbers were greater, the correlation coefficients were also calculated between the number of weekly deaths and the meteorological factors in the corresponding week and each of the two preceding weeks, as will be described more fully later.

Before discussing the correlations found, the varying mortality from the two diseases throughout the year may be referred to briefly as the figures for the four Scottish cities and London are available. The seasonal incidence is

Table I.

*Showing the annual death-rates\* in each month and the proportionate mortality in each quarter of the year from bronchitis and pneumonia in children under five years of age in London, Glasgow, Edinburgh, Aberdeen and Dundee.*

Month	Glasgow		Edinburgh		Aberdeen		Dundee		London	
	Death-rates	Proportion of deaths in four quarters of year	Death-rates	Proportion of deaths in four quarters of year	Death-rates	Proportion of deaths in four quarters of year	Death-rates	Proportion of deaths in four quarters of year	Death-rates	Proportion of deaths in four quarters of year
Jan.	16.75	31.44 ± .12	10.63	31.32 ± .27	10.42	35.34 ± .38	16.16	33.35 ± .31	8.41	34.26 ± .08
Feb.	17.47		11.37		10.12		14.82		8.28	
Mar.	16.14		9.94		9.14		12.34		8.37	
Apr.	15.03	24.24 ± .11	9.04	22.45 ± .24	7.39	20.61 ± .32	11.16	21.38 ± .27	6.46	19.70 ± .01
May	13.04		7.50		5.31		8.47		4.59	
June	10.75		6.36		4.61		8.14		3.36	
July	8.11	14.34 ± .09	5.12	13.66 ± .20	3.44	11.48 ± .25	5.83	12.84 ± .22	2.91	11.56 ± .06
Aug.	6.93		4.30		2.92		5.15		2.68	
Sept.	7.93		4.51		3.28		5.69		2.87	
Oct.	12.51	29.98 ± .12	7.21	32.56 ± .27	5.00	32.57 ± .38	9.66	32.43 ± .31	6.08	34.48 ± .08
Nov.	17.24		12.84		10.03		14.64		9.61	
Dec.	18.28		13.16		12.33		17.83		9.52	

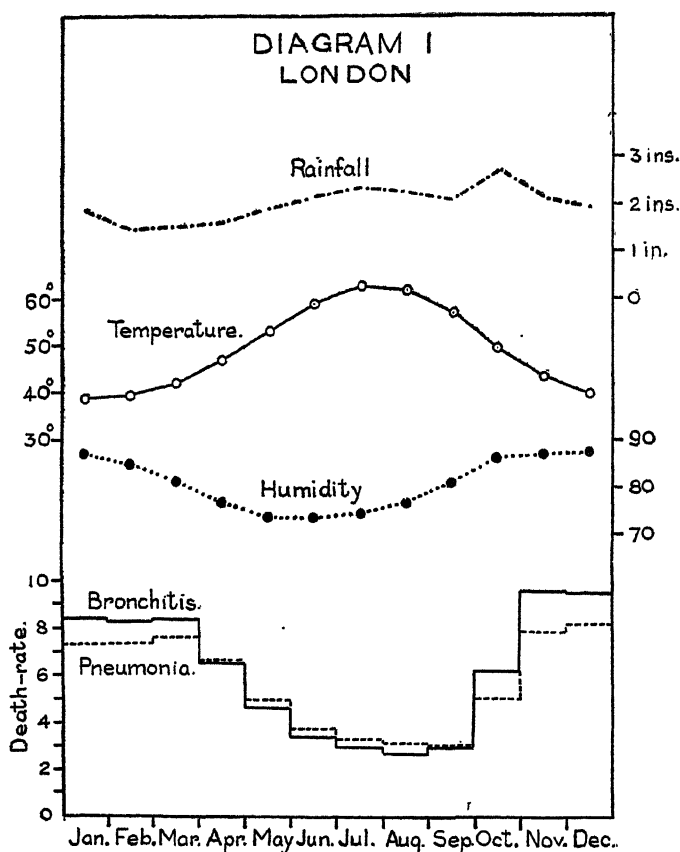
  

<i>Pneumonia.</i>										
Jan.	9.91	29.86 ± .15	5.26	29.78 ± .36	3.97	32.27 ± .56	7.44	32.90 ± .44	7.38	32.81 ± .09
Feb.	10.71		5.31		4.19		7.39		7.36	
Mar.	9.55		5.54		4.14		6.54		7.65	
Apr.	9.05	23.93 ± .14	4.67	24.26 ± .34	3.79	23.77 ± .51	6.03	24.47 ± .40	6.53	22.31 ± .08
May	8.24		4.32		2.85		5.50		4.92	
June	6.88		4.14		2.43		4.36		3.78	
July	5.40	14.43 ± .12	3.43	15.82 ± .29	2.11	14.58 ± .42	4.01	15.13 ± .34	3.36	13.89 ± .06
Aug.	4.15		2.70		1.44		3.18		3.11	
Sept.	5.03		2.43		2.01		2.64		3.00	
Oct.	8.62	31.78 ± .16	3.76	30.13 ± .36	2.73	29.38 ± .54	4.33	27.50 ± .42	5.02	31.00 ± .09
Nov.	11.73		5.76		3.96		6.31		7.98	
Dec.	11.75		6.78		4.61		7.22		8.16	

\* Calculated from the average number of deaths in the respective months for 55 years (50 years in London) corrected for increase of population, and the population under 5 years in 1911.

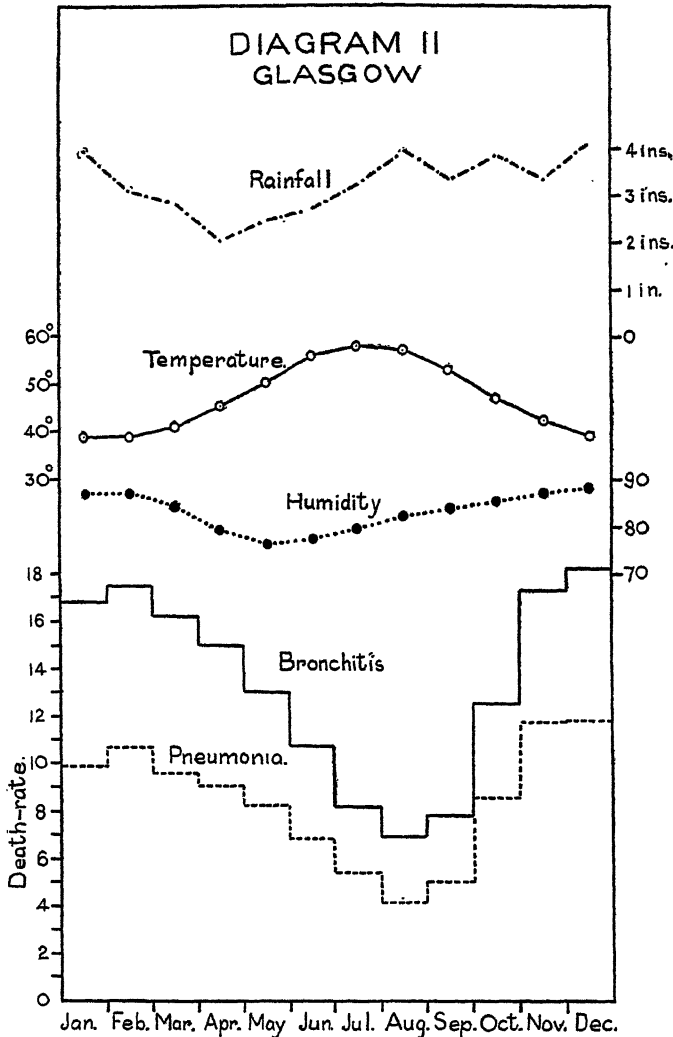
# 154 *Influence of Weather Conditions on Mortality*

very evident in both bronchitis and pneumonia, both diseases being most fatal in the first and last quarters of the year and least fatal in the third quarter. Table I shows, for each of the diseases in each of the Scottish cities and in London, the percentage of fatal cases occurring in each quarter of the year taking the average of all the 55 years. In the second quarter of the year there seems to be a slightly higher proportionate mortality from pneumonia than from bronchitis in Edinburgh, Aberdeen, Dundee and London



but not in Glasgow. The differences are not large, but, in view of the size of their probable errors, may be regarded as statistically significant. It is said to be a feature of pneumonia that its prevalence is prolonged further into the spring than that of bronchitis and there appears to be in the data for all the cities, except Glasgow, evidence in support of this view. The variation in mortality throughout the year is shown in greater detail in the annual death-rates for each month from bronchitis and pneumonia which are given in Table I. Graphs have also been drawn for London, Glasgow and Dundee to illustrate the seasonal incidence of the mortality from the respective diseases in relation to the corresponding mean temperature, relative humidity

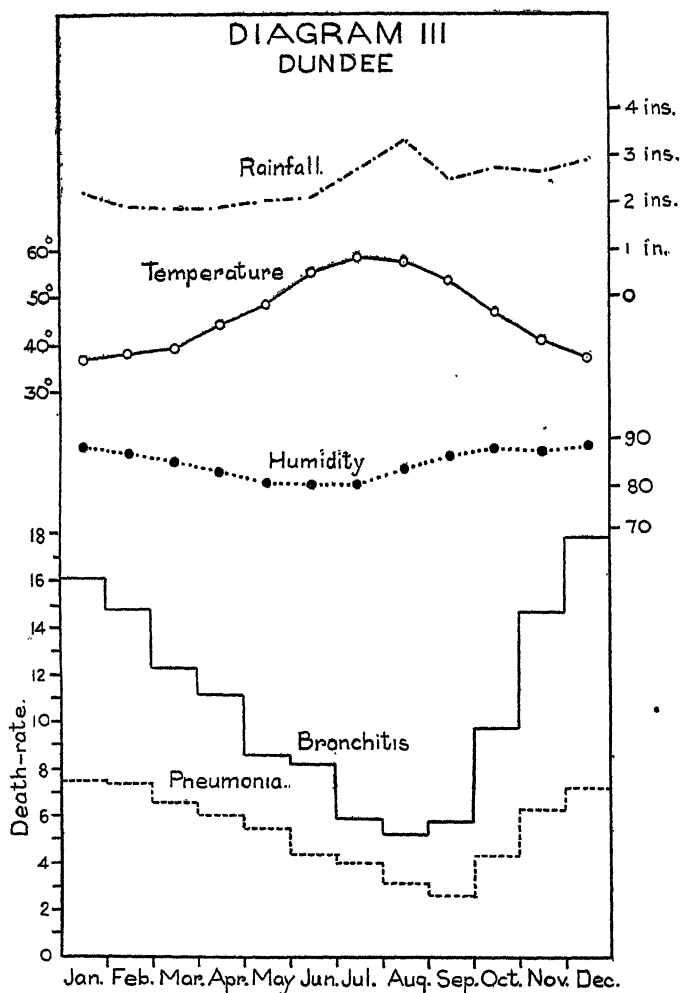
and mean rainfall (Diagrams I, II and III). The death-rates have been calculated from the average number of deaths corrected for the increase of the population for approximately 50 years, and from the populations in the respective cities in 1911. In the four Scottish cities the maximum death-rate from bronchitis is found in December, that in London in November, and the



minimum in all in August. As regards pneumonia, the maximum death-rate is found in December in Glasgow, Edinburgh, Aberdeen and London, and in January in Dundee, while the minimum death-rate is found in all the cities in August or September. There is thus abundant evidence in the data that the weather conditions obtaining in the winter months are accompanied by an increased mortality from both bronchitis and pneumonia and it now

remains to be seen if the method of correlation will serve to differentiate the most potent predisposing factors amongst those under review.

The coefficients of correlation found between the number of fatal cases in each month from the respective diseases and the two diseases combined on the one hand, and the several meteorological factors under consideration on the other, are shown in Table II and the subsequent tables. For the



numerous correlation coefficients calculated, the probable errors have not all been tabulated as it seems sufficient to state that, if the attainment of a value not less than three times its probable error, the conventional standard, be regarded as the criterion of statistical significance of a correlation coefficient, with 54 years' statistics such as are available for Glasgow, Edinburgh and Aberdeen, a coefficient requires to be about 0.25 and with 45 years' statistics,

as are available for London and Dundee, 0.30 in value before it can be stated with any degree of certainty that any real significance should be attached to, or any legitimate conclusions drawn from it. A short discussion on the relationships found will be given for each disease separately beginning with the monthly data.

Table II.

*Showing the correlation in the several months of the year between the number of monthly deaths from bronchitis in children under five years of age in Glasgow, Edinburgh, Aberdeen, Dundee and London, respectively, and the corresponding monthly mean temperature.*

Month	Glasgow (1857- 1910)	Glasgow (using differences from mean of 5 years)	Edinburgh (1857- 1910)	Aberdeen (1857- 1910)	Dundee (1866- 1910)	Mean of 4 coeffi- cients for Scottish cities	London (1876- 1919)
	(1)	(1 a)	(2)	(3)	(4)	(5)	(6)
January	-.247	-.296	-.125	+0.035	-.213	-.138	-.252
February	-.181	-.329	-.021	+0.028	-.015	-.047	-.186
March	-.138	-.242	-.151	-.073	+0.103	-.065	-.234
April	+0.150	—	+0.198	-.256	+0.291	+0.096	-.259
May	-.131	—	+0.031	+0.032	-.173	-.060	-.564
June	-.075	—	+0.224	+0.072	+0.167	+0.097	-.023
July	+0.092	—	-.069	+0.112	+0.128	+0.066	-.196
August	-.019	—	-.088	+0.177	+0.187	+0.064	-.325
September	-.139	—	+0.088	+0.126	-.016	+0.015	-.014
October	-.148	+0.039	-.158	-.121	+0.016	-.103	-.348
November	-.321	-.064	-.064	+0.285	-.034	-.034	-.124
December	-.290	-.293	-.221	-.067	-.334	-.228	-.382
Mean of coeffi- cients for six months (first 3 and last 3 of year)	-.221	-.198	-.123	+0.015	-.080	—	-.254
Mean of coeffi- cients for 12 months	-.121	—	-.030	+0.029	+0.009	—	-.242

For Glasgow, Edinburgh and Aberdeen with 54 years' statistics and for Dundee and London with 45 years' statistics, correlation coefficients require to be approximately equal to 0.250 and 0.300 respectively, to be regarded as statistically significant. The same standard applies to the succeeding tables.

*Mortality from bronchitis and temperature—mean and mean minimum.*

The coefficients of correlation between the number of monthly deaths from bronchitis and the two temperature records—mean and mean minimum—have all been calculated and are shown in Tables II and III but it is obvious, from a survey of the coefficients that those for mean minimum temperature exhibit in general a similar trend to, and would suggest the same conclusions as, those for the mean temperature. While the minimum temperature coefficients may not infrequently be a little greater than the corresponding values shown for the mean temperature it will be sufficient to confine the discussion largely to the relationship of bronchitis to the latter making only occasional reference to mean minimum temperature where such seems advisable.



If cold weather predisposes to the onset of bronchitis, as is generally believed, then there is probably a level or critical point below which the temperature must fall before it has any injurious influence, or its effect is shown in an increased mortality from the disease. As this low temperature level or threshold is more likely to be attained and more commonly and more extensively transgressed in the colder months than in the summer when it is probably rarely reached on the average, the coefficients of correlation in the winter months are presumably a more accurate index of the effect of the fall of temperature on the death-rate. Though the coefficients for all the months have been tabulated, special attention will thus be directed to the coefficients for the colder months (and in the weekly data to those for the colder weeks)

Table III.

*Showing the correlation in the several months of the year between the number of monthly deaths from bronchitis in children under five years of age in Glasgow, Edinburgh, Aberdeen and Dundee, respectively, and the corresponding mean minimum temperature.*

Month	Glasgow (1)	Edinburgh (2)	Aberdeen (3)	Dundee (4)	Mean of 4 coefficients for Scottish cities (5)
January	-.292	-.116	+·032	-.213	-.147
February	-.228	-.110	+·100	+·045	-.048
March	-.227	-.196	-.026	+·070	-.095
April	+·023	+·179	-.130	+·407	+·120
May	-.181	-.110	+·176	-.201	-.079
June	-.214	-.056	+·045	+·139	-.022
July	-.042	-.221	+·206	-.028	-.021
August	-.207	-.186	+·204	+·174	-.004
September	-.257	-.007	-.008	-.118	-.098
October	-.160	-.132	-.112	+·126	-.070
November	-.408	-.147	+·338	+·010	-.052
December	-.388	-.211	+·004	-.344	-.235
Mean of coefficients for six months (first 3 and last 3 of year)	-.284	-.152	+·056	-.051	—
Mean of coefficients for 12 months	-.215	-.109	+·069	+·006	—

which are, on the average, greater than and more regular in value than those for the warmer periods of similar duration.

For each of the four Scottish cities, the mean values of the correlation coefficients between each pair of variables for the whole series of twelve months and for the six colder months—the first three and the last three of the year—were computed. The mean values of the corresponding coefficients for each month of the year in the four cities were also determined. On comparing the coefficients, it is found that considerable variation occurs from month to month in those for each city and that there does not appear to be much evidence of agreement in corresponding months in the different cities. While the values of the correlation coefficients for the several months in Glasgow are with two exceptions negative in sign, the values in the other three towns

change irregularly from positive to negative in different months of the year. These values are, however, most frequently within the range  $+0.2$  and  $-0.2$  and are probably statistically insignificant. While the regular sequence of negative coefficients for the different months in Glasgow would suggest the tendency to an inverse relationship between the number of deaths from bronchitis and the monthly mean temperature, a relationship which is perhaps still closer between bronchitis and the monthly mean minimum temperature, it cannot be stated that there is definite evidence that such an association obtains in the other three towns. The inverse relationship described is, as would be anticipated, more clearly indicated in the coefficients for the first three and last three months of the year in Glasgow. While the correlation coefficients for January and December in Dundee and that for December in Edinburgh are negative in sign and exceed  $0.2$  in value, the coefficients for Aberdeen in these months are obviously insignificant. The correlation coefficients between the number of deaths from bronchitis and the mean temperature for the different months in London, though they show considerable variation in value, are uniformly negative in sign. The mean value of the coefficients for all the months is  $-0.242$ , and for the first and last three months  $-0.254$ . This suggests a tendency towards an inverse association between the number of deaths from bronchitis and the mean temperature of the corresponding month, the lower the mean temperature, the greater the number of deaths; this seems to support the conclusion that would be drawn from the coefficients calculated from the Glasgow data.

On investigation, there appeared to be some evidence of an approximately five yearly periodicity in the figures for bronchitis deaths in Glasgow, and it seemed possible that this might have some effect in determining the magnitude of the correlation coefficient. In an attempt to exclude this possible influence, correlation coefficients were calculated for each of the six colder months for the period of 50 years between the deviations from the number of deaths from bronchitis in the month in any year and the average number of deaths in that month in the five adjacent years, of which the given year is the centre and the mean temperature of the corresponding month (Table II, col. 1 a). The correlation coefficients remained negative and statistically significant in four out of the six months, indicating that the correlation found between the mortality and the mean temperature is real and is not to be accounted for by the periodicity in the figures.

*Mortality from pneumonia and temperature—mean and mean minimum.*

The coefficients of correlation between the monthly deaths from pneumonia in children and the corresponding mean and mean minimum temperatures for the four Scottish towns were calculated and the two sets of values for these cities and the values for mean temperature in London are shown in Tables IV and V. It will be seen that the mean values of the twelve monthly coefficients and of those for the six colder months of the year in each of the four

Table IV.

*Showing the correlation in the several months of the year between the number of monthly deaths from pneumonia in children under five years of age in Glasgow, Edinburgh, Aberdeen, Dundee and London, respectively, and the corresponding monthly mean temperature.*

Month	Glasgow (1)	Glasgow (using differences from mean of 5 years) (1 a)	Edinburgh (2)	Aberdeen (3)	Dundee (4)	Mean of 4 coeffi- cients for Scottish cities (5)	London (6)
January	-.185	-.302	+.111	+.139	+.072	+.034	+.156
February	-.391	-.467	-.104	-.004	-.193	-.173	-.131
March	-.111	-.188	+.166	-.009	+.153	+.050	-.154
April	-.408	—	-.161	-.161	-.115	-.211	+.098
May	+.027	—	-.248	+.066	-.026	-.078	-.199
June	-.129	—	-.335	-.137	-.012	-.153	-.054
July	-.055	—	-.308	-.120	-.024	-.127	+.134
August	+.025	—	+.068	-.068	+.029	+.014	-.059
September	+.210	—	+.089	+.180	+.125	+.151	+.168
October	+.103	+.105	+.045	+.014	-.059	+.026	+.052
November	+.161	-.130	+.238	+.314	+.225	+.235	-.191
December	-.020	-.204	+.034	+.040	-.087	-.008	-.046
Mean of coeffi- cients for six months (first 3 and last 3 of year)	-.074	-.198	+.082	+.082	+.019	—	-.052
Mean of coeffi- cients for 12 months	-.064	—	-.034	+.010	+.007	—	-.019

Table V.

*Showing the correlation in the several months of the year between the number of monthly deaths from pneumonia in children under five years of age in Glasgow, Edinburgh, Aberdeen and Dundee, respectively, and the corresponding mean minimum temperature.*

Month	Glasgow (1)	Edinburgh (2)	Aberdeen (3)	Dundee (4)	Mean of 4 coefficients for Scottish cities (5)
January	-.113	+.095	+.108	-.017	+.018
February	-.398	-.094	-.089	-.217	-.200
March	-.033	+.153	-.063	+.093	+.038
April	-.206	-.112	-.147	-.299	-.191
May	+.132	-.258	-.173	-.006	-.076
June	-.027	-.180	-.115	-.059	-.095
July	+.042	-.347	-.140	-.066	-.128
August	+.110	+.024	-.189	-.036	-.023
September	+.169	+.046	+.072	-.068	+.055
October	+.162	+.042	-.033	+.157	+.082
November	+.218	+.279	+.233	+.208	+.247
December	+.119	-.031	-.003	-.016	+.017
Mean of 6 coefficients (first 3 and last 3 of year)	+.008	+.074	+.034	+.035	—
Mean of 12 coefficients	+.015	-.032	-.041	-.027	—

Scottish towns are less than 0.1, showing that no consistent relationship, either inverse or direct, between the number of deaths and the temperature is evident, on the average, in the months throughout the year. For the mean temperature, the series of means of the corresponding coefficients for each month in the four towns are small in value and variable in sign; in only two months, April and November, does the mean value exceed 0.2, and of these, the sign is negative in the former and positive in the latter. The correlation coefficients between the monthly mortality from pneumonia and mean temperature in London vary irregularly in sign and in no month attain a value as high as 0.2. While the coefficients for a few individual months in Glasgow and Edinburgh suggest a tendency therein to an inverse relationship between pneumonia and mean temperature, the only conclusion that can be drawn from the coefficients as a whole is that, if any consistent relationship exists between the mortality from pneumonia and the temperature that prevails, it is not disclosed by correlating the monthly deaths with the monthly mean or minimum temperature.

*Mortality from respiratory diseases and temperature.*

With the view of excluding the influence, on the correlation coefficient, of the transference of cases from bronchitis to pneumonia in the data that has occurred in recent years, the correlation coefficients between the monthly

Table VI.

*Showing the correlation in the several months of the year between the number of deaths from respiratory diseases in children under five years of age in Glasgow, Edinburgh, Aberdeen and Dundee, respectively, and the corresponding mean monthly temperature.*

Month	Glasgow (1)	Glasgow (using differences from mean of 5 years) (1 a)	Edinburgh (2)	Aberdeen (3)	Dundee (4)	Mean of 4 coefficients (5)
January	-.374	-.360	-.098	+.050	-.135	-.139
February	-.354	-.412	-.169	+.006	-.086	-.151
March	-.243	-.197	-.050	+.018	+.128	-.037
October	-.079	+.069	-.069	-.006	-.015	-.042
November	-.213	-.078	-.047	+.339	+.016	+.024
December	-.394	-.343	-.137	-.227	-.356	-.279
Mean of 6 coefficients	-.276	-.220	-.095	+.030	-.075	—

deaths from bronchitis and pneumonia summed together, and the mean temperature of the corresponding month have been calculated for the six colder months of the year for each of the four Scottish towns (Table VI). While some of the coefficients for the months in Glasgow appear to be statistically significant and the mean of the six is -0.276, suggesting that the number of deaths from the respiratory diseases in children in this city is inversely

associated with the mean temperature, only one or two of the coefficients for the months in the other three cities can be regarded as significant.

*Mortality from bronchitis and relative humidity.*

The interpretation of the correlation coefficients between these variables in the different cities, as shown in Table VII, presents some difficulty. For Glasgow alone, the problem, at the first glance, seems simple as the coefficients for the several months, are uniformly positive in sign and all exceed 0·3 in value; that for February being as high as 0·7 and that for November 0·4. They may thus all be regarded as statistically significant and seem to indicate a definite, direct association between the degree of relative humidity and the

Table VII.

*Showing the correlation in the several months of the year between the number of monthly deaths from bronchitis in children under five years of age in Glasgow, Edinburgh, Aberdeen, Dundee and London, respectively, and the corresponding mean monthly relative humidity.*

Month	Glasgow (1)	Glasgow (using differences from mean of adjacent 5 years) (1 a)	Edinburgh (2)	Aberdeen (3)	Dundee (4)	Mean of 4 coeffi- cients for Scottish cities (5)	London (6)
January	+·343	+·014	+·331	+·235	-·193	+·179	-·199
February	+·693	+·152	+·048	+·417	-·068	+·273	-·003
March	+·361	-·143	-·017	+·190	-·213	+·080	-·419
April	+·485	—	+·081	+·346	-·161	+·188	+·170
May	+·419	—	-·315	+·197	-·418	-·029	-·060
June	+·527	—	-·027	+·051	-·233	+·080	+·128
July	+·393	—	+·008	+·174	-·123	+·113	-·037
August	+·428	—	-·046	+·164	+·010	+·139	+·075
September	+·511	—	-·071	+·157	-·305	+·073	+·043
October	+·390	-·034	-·184	—	-·206	—	-·269
November	+·399	-·043	-·046	-·083	-·212	+·015	-·324
December	+·324	-·211	+·056	+·083	-·259	+·051	-·071
Mean of coeffi- cients for six months (first 3 and last 3 of year)	+·418	-·044	+·031	+·140	-·192	—	-·214
Mean of coeffi- cients for 12 months	+·439	—	-·015	+·161	-·198	—	-·081

mortality from bronchitis—the higher the relative humidity, the greater the mortality from the disease. The mean of the coefficients for the twelve months is + 0·439 and that for the six colder months, including the first three and the last three of the year + 0·418. This is less than the mean of the twelve coefficients as some of the coefficients for the intermediate months are relatively large. The correlation coefficients for Aberdeen are, with one exception, positive, and, though only two or three of them may be regarded as definitely significant, the uniformity in the sign of the coefficients seems to suggest the

existence of a tendency towards a direct association between a high relative humidity and a high death-rate from bronchitis in children. For Dundee, however, the coefficients for all the months, with one exception, are negative and exceed 0.20 in value in half the months and 0.30 in two instances; while those for Edinburgh vary irregularly in sign and exceed 0.25 in value in two months only, viz., in January where the coefficient is positive and in May where it is negative. The coefficients for the different months in London are generally negative though some are positive. In two months, namely, March and November, the coefficients exceed 0.30 in value and are negative in sign. There is thus no evidence in these last named towns of a direct association between the mortality from bronchitis and the index of relative humidity, such as might be concluded from the Glasgow and Aberdeen data; if the coefficients suggest any association at all, it is an inverse relationship.

*Mortality from pneumonia and relative humidity.*

If discussion were confined to the relationship between these variables as seen in the Glasgow data, the interpretation would be comparatively easy as the correlation coefficients for all the months are negative in sign with one exception, in six of the months exceed 0.25 in value and are statistically significant (Table VIII). This would suggest that the mortality from pneumonia is inversely associated with the degree of humidity, *i.e.* the relatively

Table VIII.

*Showing the correlations in the several months of the year between the number of monthly deaths from pneumonia in children under five years of age in Glasgow, Edinburgh, Aberdeen, Dundee and London, respectively, and the corresponding mean monthly relative humidity.*

Month	Glasgow (1)	Glasgow (using differences from mean of 5 years)	Edinburgh	Aberdeen	Dundee	Mean of 4 coeffi- cients for Scottish cities	London
	(1)	(1 a)	(2)	(3)	(4)	(5)	(6)
January	-.357	-.039	-.374	-.182	+.235	-.170	-.153
February	-.109	+.079	-.078	-.356	+.221	-.081	+.053
March	-.416	-.156	-.107	-.043	+.250	-.079	+.171
April	-.315	—	-.082	-.283	+.242	-.110	-.121
May	+.020	—	+.180	-.041	+.344	+.126	+.007
June	-.280	—	-.170	+.130	+.181	-.035	-.007
July	-.167	—	-.056	+.002	+.177	-.011	-.416
August	-.201	—	+.035	+.237	+.391	+.116	-.042
September	-.254	—	+.241	-.189	+.271	+.017	-.211
October	-.298	-.099	-.071	-.117	+.050	-.109	+.149
November	-.207	+.113	-.254	-.254	+.303	-.103	+.078
December	-.171	+.030	-.104	-.200	+.416	-.015	-.040
Mean of six co- efficients (first 3 and last 3 of year)	-.260	-.012	-.165	-.192	+.246	—	+.043
Mean of 12 co- efficients	-.230	—	-.070	-.108	+.257	—	-.044

drier the atmosphere may be the greater the mortality. This suggestion receives some support from the coefficients found for Aberdeen which are, with a few exceptions, negative in sign and in some months exceed 0.25 in value. The monthly coefficients for Dundee are, however, as consistently positive, with an equal claim to significance and suggest that the prevalence of pneumonia is favoured by a relatively humid atmosphere. The majority of the coefficients for Edinburgh are negative in sign but only two, viz. those for January and November can be considered to approach a significant value. About half the coefficients for London are negative and the other half positive, but only one, viz. that for July, which is negative, can be considered significant. It is evident, therefore, that while humidity would appear to have an inverse association with the mortality from pneumonia as is shown in the results for Glasgow, a view which is, to some extent, supported by the coefficients found for Aberdeen, this apparent association is not confirmed by the coefficients found for Dundee. It is worthy of note that, while the Glasgow data show a positive correlation between bronchitis and relative humidity in the several months, the correlation between pneumonia and this meteorological factor is as consistently negative. This would appear to indicate that, while a relatively moist atmosphere predisposes to bronchitis, the onset of pneumonia, on the other hand, is favoured by a relatively dry atmosphere. This difference, if real, may be regarded as, to some extent, in favour of the view that the two diseases are distinct, and in opposition to that which attributes nearly all deaths from bronchitis under five years of age to broncho-pneumonia in which the physical signs are indefinite.

*Mortality from respiratory diseases and relative humidity.*

The correlation coefficients between the combined figures for the monthly deaths from bronchitis and pneumonia and the relative humidity in the six colder months for each of the four Scottish cities are shown in Table IX. For Glasgow, five of the six coefficients appear to be statistically significant and the mean value of the six is + 0.315, suggesting a definite tendency for a high relative humidity to be associated with a high death-rate from the respiratory diseases. This direct relationship was also found between bronchitis and relative humidity, and the deaths from bronchitis being more numerous than those for pneumonia, the preponderance seems to determine the direct relationship for the two diseases combined, as the relationship suggested between the deaths from pneumonia and the mean relative humidity is inverse. The values of the monthly coefficients for the other three cities are practically all statistically insignificant and the mean values for each city are less than 0.1.

As it seemed possible that the correlation coefficients between bronchitis and relative humidity, pneumonia and relative humidity and respiratory diseases and relative humidity for the Glasgow data, which were relatively high in comparison with those found for the other Scottish cities, might be

determined, in some degree, by the five yearly periodicity which appeared to exist in the mortality figures, other correlation coefficients were calculated, for the first three and last three months of the year, between the differences in the number of deaths, (1) from bronchitis, (2) from pneumonia, (3) from respiratory diseases, in any month in one year, and the average number of deaths in the corresponding month in the adjacent five years of which the given year is the centre, and the relative humidity in the corresponding month. The correlation coefficients found in this manner from the bloxamed monthly figures between bronchitis and relative humidity, pneumonia and relative humidity, and respiratory diseases and relative humidity, were much less in value than those found from the actual figures and were not statistically

Table IX.

*Showing the correlation in the several months of the year between the number of deaths from respiratory diseases in children under five years of age in Glasgow, Edinburgh, Aberdeen and Dundee, respectively, and the corresponding mean monthly relative humidity.*

	Glasgow (using differences from mean of adjacent 5 years)					Mean of 4 coefficients
Month	Glasgow (1)	(1 a)	Edinburgh (2)	Aberdeen (3)	Dundee (4)	(5)
January	+·434	+·019	+·159	+·095	-·142	+·137
February	+·501	+·120	+·067	+·123	+·086	+·194
March	+·271	-·139	-·072	+·294	-·054	+·110
October	+·150	-·054	+·171	-·024	-·154	+·036
November	+·295	+·035	-·151	-·150	-·003	-·002
December	+·241	-·119	-·022	-·076	-·143	000
Mean of coefficients for six months (first 3 and last 3 of year)	+·315	-·023	+·025	+·044	-·068	—

significant for any month, the averages for the six coefficients in the three cases being - 0·044, - 0·012 and - 0·023, respectively (cols. 1 a in Tables VII, VIII and IX). While this would appear to indicate that the correlation coefficients between the mortality from bronchitis, pneumonia and respiratory diseases, respectively, and relative humidity in Glasgow are due to the periodicity in the mortality data, the effect of which is probably eliminated by bloxaming the data and correlating the differences, this does not seem to be an adequate explanation of the reduction in the coefficients that takes place by using this method, as it will be recollected that the correlation coefficients between the same averaged data and mean temperature, remain significant. As there is no definite periodicity visible in the figures for relative humidity for any month in the series of years to account for the reduced values of the coefficients, it is possible that the application of the bloxaming process to the mortality figures results in the smoothing out of the deviations and that the consequent reduction in the coefficient is, to some extent, for-



tuitous. A high relative humidity in Glasgow may be indicative of the presence of fog or other muggy, unpleasant conditions which, as is well known, are more prevalent in this city than in the other Scottish cities and may be responsible, in part, for the differences found in their coefficients of correlation.

*Mortality from bronchitis and rainfall.*

The coefficients of correlation between the monthly mortality from bronchitis and the amount of rainfall in each month in the different towns are shown in Table X. The coefficients for the several months in each town vary irregularly in sign and show considerable variation in value. The means of

Table X.

*Showing the correlation in the several months of the year between the number of monthly deaths from bronchitis in children under five years of age in Glasgow, Edinburgh, Aberdeen, Dundee and London, respectively, and the corresponding mean monthly rainfall.*

	Glasgow	Edinburgh	Aberdeen	Dundee	Mean of 4 coefficients for Scottish cities	London
Month	(1)	(2)	(3)	(5)	(5)	(6)
January	+·321	+·151	-·032	+·125	+·141	-·135
February	+·238	-·141	+·153	+·203	+·113	-·192
March	-·148	-·235	+·087	-·112	-·102	-·431
April	+·025	+·141	-·011	+·187	+·086	+·006
May	-·173	-·189	+·077	+·059	-·057	+·077
June	+·061	+·081	-·094	+·218	+·067	-·007
July	+·106	+·149	-·009	+·120	+·092	+·040
August	+·038	-·042	+·140	-·115	+·005	-·031
September	+·189	+·238	+·074	+·101	+·151	+·065
October	+·079	-·028	-·031	+·147	+·042	+·132
November	-·073	-·026	-·230	+·306	-·006	-·036
December	+·113	+·106	+·010	+·070	+·075	-·415
Means of coefficients for six months (first 3 and last 3 of year)	+·088	-·029	-·007	+·123	—	-·180
Mean of 12 coefficients	+·065	+·017	+·011	+·109	—	-·077

the coefficients for all the months in any one of the Scottish towns, and of the corresponding coefficients in the same month for the four towns rarely exceed 0·1 in value and cannot be regarded as statistically significant. The only warrantable conclusion seems to be that the average amount of monthly rainfall has no appreciable or consistent influence in determining the mortality from bronchitis in children.

*Mortality from pneumonia and rainfall.*

A survey of the coefficients of correlation between the number of deaths from pneumonia in each month and the registered rainfall in the corresponding month for the several towns, as given in Table XI, shows no evidence of a consistent relationship between them. There is a suggestion, from the values of the coefficients found for some individual months, in the Scottish cities

that, in these months, there may be a tendency towards an inverse association between the mortality from pneumonia and the amount of rainfall—the less the rainfall the greater the mortality—but there is no definite evidence of this in the coefficients for the months generally.

The correlation coefficients that have been found between the number of deaths in each month from bronchitis and respiratory diseases, respectively, and the monthly mean temperature in Glasgow and London, while suggestive of the existence of some inverse relationship between these variables, indicating a tendency for the mortality to be increased with the prevalence of colder weather, are perhaps not of sufficient magnitude to warrant the conclusion that such a tendency is unequivocally present. In addition to the

Table XI.

*Showing the correlation in the several months of the year between the number of monthly deaths from pneumonia in children under five years of age in Glasgow, Edinburgh, Aberdeen and Dundee, respectively, and the corresponding mean monthly rainfall.*

Month	Glasgow	Edinburgh	Aberdeen	Dundee	Mean of 4 coefficients for Scottish cities
	(1)	(2)	(3)	(4)	(5)
January	-.366	-.083	-.058	-.166	-.168
February	-.053	-.245	-.198	-.100	-.149
March	-.131	+.082	+.142	-.006	+.022
April	+.022	+.195	+.064	+.002	+.071
May	+.090	+.009	+.015	+.067	+.045
June	-.021	+.092	-.064	+.104	+.028
July	+.055	-.086	+.032	-.127	-.032
August	-.100	+.049	-.118	-.041	-.053
September	+.045	-.221	-.231	-.220	-.157
October	-.046	+.074	-.112	-.039	-.031
November	-.306	-.262	-.160	+.186	-.136
December	-.119	+.125	-.048	+.135	+.023
Mean of coefficients for six months (first 3 and last 3 of year)	-.170	-.052	-.072	+.002	—
Mean of 12 coefficients	-.078	-.023	-.061	-.017	—

possibility that some fatal cases, owing to the variable duration of the disease, may not be correlated with the appropriate temperature coincident with their onset and so may tend to obscure the real relationship between the mortality from the disease and the temperature, another possible source of inaccuracy is that, in correlating the monthly mean and minimum temperature with the mortality from the diseases, the effect of sudden changes of temperature of such short duration as to influence only to a small extent, if at all, the monthly means is not elicited; although it would appear, from clinical experience and observation, that these sudden or less prolonged changes may exert a considerable influence on the mortality from and the prevalence of bronchitis and pneumonia. It seemed probable that more detailed information of the influence of the temperature accompanying the onset of the diseases might be obtained from the correlations between the mortality from the two diseases

in the shorter period of a week and the mean temperature of the corresponding week as well as that of each of the two preceding weeks. These, as has been mentioned, have been calculated from the data for London for the first thirteen and last thirteen weeks of the year only, as it was in this period of the year that the real effect of reduced temperature on the respiratory death-rate was likely to be shown. We now come to consider the relationship between the several meteorological factors and the weekly mortality data. The correlation coefficients are shown in Tables XII and XIII and the relationships found therein will be discussed briefly.

Table XII.

*Showing the correlation coefficients between the number of weekly deaths from bronchitis and respiratory diseases, respectively, amongst children under five years of age in London and the several meteorological factors under investigation, for the weeks at the beginning and end of the year.*

Week of Year	Variables No. of deaths from bronchitis and							Variables No. of deaths from respiratory diseases and	
	mean tempe- rature of cor- respond- ing week	mean tempera- ture of preced- ing week	mean tempe- rature of two weeks before	mean minimum tempera- ture of same week	relative humidity of same week	relative humidity of pre- ceding week	recorded rainfall of same week	mean tempera- ture of the same week	mean tempe- rature of the pre- ceding week
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
1st	-.402	-.309	-.513	-.493	+.152	+.059	+.006	-.394	-.411
2nd	-.297	-.470	-.177	-.170	+.390	+.142	-.207	-.215	-.487
3rd	-.078	-.395	-.370	-.090	+.139	+.137	-.128	-.025	-.258
4th	-.008	-.181	-.290	-.059	-.096	+.140	-.092	+.075	-.157
5th	+.026	-.141	-.225	-.034	+.457	+.109	+.063	+.045	-.148
6th	-.083	-.039	-.090	-.112	+.312	+.386	-.119	+.026	-.106
7th	-.095	-.242	-.111	+.181	+.234	+.180	+.171	-.032	-.182
8th	-.178	-.252	-.302	-.340	+.357	+.191	-.189	-.375	-.256
9th	-.185	-.313	-.322	-.142	+.047	+.373	-.158	-.186	-.417
10th	-.298	-.324	-.269	-.370	-.067	+.132	-.386	-.410	-.321
11th	-.214	-.412	-.257	-.259	-.152	-.204	-.230	+.031	-.386
12th	-.018	-.174	-.347	-.017	+.178	-.254	-.119	+.010	-.148
13th	-.169	-.182	-.186	-.177	+.017	+.083	-.021	-.235	-.191
40th	-.146	-.248	.000	-.135	+.282	+.242	+.094	-.329	-.247
41st	-.187	-.273	-.305	-.144	-.070	+.197	+.004	-.243	-.267
42nd	-.362	-.220	-.175	-.359	+.251	-.008	+.115	-.340	-.206
43rd	-.114	-.421	-.186	-.020	+.109	+.149	+.114	-.044	-.369
44th	-.337	-.210	-.281	-.149	+.093	+.032	-.086	-.363	-.152
45th	-.353	-.511	-.213	-.309	+.175	+.076	+.019	-.296	-.532
46th	-.096	-.401	-.305	-.139	+.147	+.106	-.087	-.149	-.429
47th	-.192	-.321	-.345	-.126	+.117	+.053	-.019	-.398	-.339
48th	-.351	-.360	-.176	-.313	-.017	-.026	-.088	-.311	-.452
49th	-.441	-.376	-.189	-.327	-.331	-.084	-.227	-.379	-.450
50th	-.467	-.522	-.306	-.535	+.126	-.364	-.281	-.424	-.487
51st	-.324	-.633	-.481	-.234	+.236	+.190	-.225	-.236	-.587
52nd	-.302	-.582	-.412	-.324	+.192	+.229	-.042	-.192	-.539
Mean of coeffi- cients for 26 weeks (first 13 and last 13 of year)	-.218	-.327	-.263	-.200	+.126	+.087	-.081	-.207	-.328
Means of coeffi- cients for 52 weeks	-.127	—	—	-.115	+.175	—	-.022	—	—

Table XIII.

*Showing the correlation coefficients between the number of weekly deaths from pneumonia amongst children under five years of age in London, and the several meteorological factors under investigation for the weeks at the beginning and end of the year.*

Week of Year	Variables						
	No. of deaths from pneumonia and						
	mean temperature of corresponding week	mean temperature of the preceding week	mean temperature of two weeks before	mean minimum temperature of same week	relative humidity of the same week	relative humidity of the preceding week	recorded rainfall of same week
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
	<i>r</i>	<i>r</i>	<i>r</i>	<i>r</i>	<i>r</i>	<i>r</i>	<i>r</i>
1st	+·038	−·160	−·033	+·090	−·308	−·437	−·036
2nd	+·165	−·258	−·218	+·031	−·129	−·272	−·039
3rd	+·075	−·013	−·127	+·107	−·088	−·198	−·218
4th	+·085	+·072	+·154	+·134	−·116	−·120	−·023
5th	−·001	−·170	−·040	−·048	−·050	−·127	−·022
6th	−·012	−·172	−·248	+·025	−·344	−·010	+·064
7th	−·172	−·174	−·108	−·204	−·256	−·217	−·198
8th	−·282	−·167	−·158	−·287	−·104	−·265	−·227
9th	−·161	−·386	−·373	−·217	−·119	−·243	−·112
10th	−·216	−·264	−·282	−·150	+·163	−·078	+·032
11th	+·128	−·367	−·257	+·082	−·032	+·077	−·088
12th	+·095	+·070	−·222	+·174	+·055	−·082	−·061
13th	−·188	−·090	+·030	−·281	−·158	−·175	+·017
40th	+·104	−·094	+·009	+·155	+·048	+·063	+·063
41st	−·103	+·065	−·059	−·091	−·057	+·106	+·015
42nd	+·130	+·110	+·159	+·207	+·271	−·062	+·151
43rd	−·036	+·016	+·113	−·072	−·096	+·033	−·002
44th	−·151	−·012	+·146	−·074	+·162	+·012	+·009
45th	+·037	−·259	−·088	+·023	+·044	−·108	−·097
46th	−·161	−·279	−·136	−·183	−·181	−·040	−·229
47th	−·487	−·288	−·251	−·517	−·286	−·346	−·142
48th	−·147	−·529	−·096	−·211	−·047	−·266	+·080
49th	−·091	−·212	−·410	−·026	−·110	−·109	−·076
50th	+·100	+·050	−·093	+·094	−·279	−·046	+·232
51st	+·113	−·224	+·134	+·120	−·074	−·248	−·216
52nd	−·002	−·103	−·034	−·059	−·309	+·016	+·021
Mean of coefficients for 26 weeks (first 13 and last 13 of year)	−·043	−·148	−·096	−·045	−·092	−·123	−·042
Mean of coefficients for 52 weeks	+·006	—	—	+·006	−·083	—	+·005

*Weekly mortality from bronchitis and temperature—mean and mean minimum.*

The correlation coefficients between the number of deaths in each week from bronchitis in children under five years of age in London, and the mean and mean minimum temperatures of the corresponding weeks are negative in about 42 out of 52, or 80 per cent., of the weeks in the year. The means of the coefficients for the 52 weeks are only −0·127 and −0·115, respectively, so that a significant inverse relationship between the mortality and the temperature is not evident generally in the weeks throughout the year. The correlation coefficients for a number of the weeks at the beginning and at the end of the year, the colder weeks, are, however, of sufficient magnitude to be

regarded as statistically significant and to indicate an inverse association between the mortality and temperature. The coefficients between the number of fatal cases in each week and the mean temperature of the preceding week and of the week before that show that the mortality is more highly correlated with the temperature of the previous week, than with the temperature of the corresponding week or that of two weeks before. The majority of the coefficients are statistically significant and the mean for the 26 weeks is  $-0.327$ . For the mortality and the temperature of two weeks before, the mean of the coefficients is  $-0.263$ , while that of the coefficients between the weekly mortality and the temperature of the corresponding week is, as has been mentioned,  $-0.218$ .

An attempt was made to obtain evidence of the influence of the sudden or less prolonged changes of temperature on the mortality from bronchitis in children by correlating the number of weekly deaths from this disease in children in London with the differences in temperature in preceding weeks and with other temperature deviations, but the values obtained did not show an appreciably closer relationship between the mortality from bronchitis and such deviations of temperature than was found between the mortality and the mean temperature of the corresponding weeks.

It is probable that some relationship, determinable by correlation, may exist between the number of deaths from bronchitis in weekly or monthly periods and the aggregated or accumulated temperature below a certain critical temperature in the same or some antecedent period of a similar duration. Absence of all information at the present time, however, as to the temperature level which may be regarded as critical for the respective diseases and the amount of work that would be necessary to determine it by trial, preclude this method of investigation.

*Weekly mortality from pneumonia and temperature—mean and mean minimum.*

The coefficients of correlation between the weekly mortality from pneumonia and the mean and mean minimum temperatures of the corresponding weeks throughout the year do not show such a high proportion of negative sign as is found for bronchitis. For both temperatures—mean and the mean minimum—the negative coefficients are about equal in number to the positive coefficients and the means for the two series of 52 are identical and practically zero, viz.  $+0.006$ . For the 26 colder weeks, the mean values of the coefficients are  $-0.043$  and  $-0.045$ . There is thus no evidence that, in general throughout the year, the mortality from pneumonia in any week is related to the mean or the mean minimum temperature of the corresponding week. On correlating the number of weekly deaths from pneumonia with the mean temperatures of each of the two preceding weeks for the same period of the year, there is no evidence that the mortality from pneumonia is more closely related to

the mean temperature of the preceding week than to the temperature of the same week or to the temperature of two weeks before.

With reference to this result, it is interesting to note that Dr Greenwood<sup>1</sup> obtained definitely significant correlation coefficients on correlating the death-rate per million from *all forms of pneumonia* in each of the first twelve and last twelve weeks of the year respectively, for a series of 40 years, 1850–1889, in London, with the mean temperature of the immediately preceding week. The coefficients for the weeks in the first quarter and last quarter of the year were  $-0.371$  and  $-0.458$  respectively. The relatively high magnitude of these values apparently depends, in large measure, on the inclusion in the mortality data of deaths of old people who succumb readily to the pneumonia which is predisposed to by the fall in temperature of the previous week.

*Weekly mortality from respiratory diseases and mean temperature.*

The correlation coefficients between the weekly deaths from the two respiratory diseases, bronchitis and pneumonia summed together, and the mean temperatures of the corresponding and the preceding week are shown in Table XII. The weekly mortality is apparently more closely related to the temperature of the preceding week than to the temperature of the corresponding week. The mean values of the two series of correlation coefficients are practically identical with those found for bronchitis and mean temperature and are apparently determined, in large measure, by the preponderance of the component figures for bronchitis over those for pneumonia in the combined totals.

*Weekly mortality from bronchitis and pneumonia and relative humidity.*

Of the series of 52 correlation coefficients between the weekly mortality from bronchitis and the relative humidity in the same week, 45 are positive and the mean is  $+0.175$ , while the mean of the coefficients for the 26 colder weeks is  $+0.126$ . Though there would appear to be, in the coefficients for a few of the weeks, some indication of the existence of a tendency towards a direct association between the mortality from bronchitis and the degree of relative humidity, the reverse of what has been found for bronchitis and mean temperature, there is no evidence of a tendency to such a relationship between the mortality from bronchitis and the relative humidities of either the corresponding or the preceding week in the weeks generally.

In contrast to what is found for bronchitis the majority of the correlation coefficients between the number of weekly deaths from pneumonia in children and the relative humidity in the corresponding week are negative. The mean of the coefficients found for the first thirteen and last thirteen colder weeks of the year is  $-0.092$ , while the mean of the coefficients between the mortality from pneumonia and the temperature of the preceding week for the same

<sup>1</sup> Greenwood, M. Report on the Pandemic of Influenza, 1918–19, Chap. VII, p. 162. The Relation between Meteorological conditions and the Death-rate from Respiratory Diseases with particular reference to Influenza. *Ministry of Health Reports*, No. 4.

period of the year is only — 0.123. There is thus no evidence that, throughout the period of the year investigated, any definite relationship exists between the weekly mortality from pneumonia and the recorded relative humidity of the corresponding or preceding week.

*Weekly mortality from bronchitis and pneumonia and mean rainfall.*

The coefficients of correlation between the weekly mortality from bronchitis and pneumonia, respectively, in London children and the rainfall in the corresponding week show no evidence that the mortality from either disease is related to the average weekly amount of rainfall.

*Mortality from bronchitis and pneumonia in registration counties of England and Wales, and mean annual temperature.*

Fortunately it has been possible to investigate the influence of the meteorological factors, temperature and rainfall, on the mortality from bronchitis and pneumonia in children under five years using other data than those described for the cities. These comprised the death-rates from bronchitis, pneumonia and respiratory diseases—the two combined—in the 55 registration counties of England and Wales for the period 1901–10, as recorded in the decennial supplement to the report of the Registrar-General of England, and the corresponding particulars for respiratory diseases in the 42 counties of England in the decade 1891–1900; the Welsh counties not being given separately for this period.

The average annual rainfall for each several county has been estimated by superimposing the outlines of the areas with different amounts of rainfall as shown on the map of the British Isles prepared by Dr H. R. Mill, Director of the British Rainfall Organisation, on another map of large size showing the boundaries of the counties. From the latter map it has been possible by the aid of the planimeter to obtain the area of each county, the areas therein with different amounts of average annual rainfall and from these to calculate the average annual fall of the county. The figures for mean annual temperature for the respective counties have been obtained by a similar method from a map prepared by Prof. A. J. Herbertson, published in Bartholomew's Meteorological Atlas. In this map the actual temperature in different places is shown and not, as is usual, the temperature reduced to sea level. It will be readily understood that the mean actual temperature calculated for a complete county from these different values, can only be accepted as a fair criterion of the climate to which the majority of the children therein are subject if these are more or less evenly distributed throughout the county as is the case in many counties of England and Wales.

The coefficients of correlation found between the mortality from bronchitis and pneumonia, respectively, and mean annual temperature in the registration counties are shown in Table XIV. It is seen that the coefficients between mean temperature and (1) bronchitis, (2) pneumonia, (3) bronchitis

Table XIV.

*Showing the correlation coefficients between the death-rates from bronchitis, pneumonia, and respiratory diseases, respectively, in children under five years of age in the registration counties of England and Wales in the decades 1901-10, and 1891-1900 and the mean annual temperature and mean annual rainfall.*

Variables	Correlation coefficient <i>r</i>
Death-rate from:	
bronchitis in 55 counties of England and Wales and mean annual temperature (1901-10)...	$-.314 \pm .082$
pneumonia .....	$-.240 \pm .086$
respiratory diseases in 55 counties of "England and Wales and mean annual temperature (1901-10) ... ..	$-.280 \pm .084$
respiratory diseases in 42 counties of England and mean annual temperature (1891-1900)	$-.350 \pm .091$
bronchitis in 55 counties of England and Wales and mean annual rainfall (1901-10) ...	$-.120 \pm .090$
pneumonia .....	$+.150 \pm .089$
respiratory diseases in 55 counties of "England and Wales and mean annual rainfall (1901-10)	$+.051 \pm .091$
respiratory diseases in 42 counties of England and mean rainfall (1891-1900) ... ..	$+.065 \pm .104$
bronchitis in 55 counties of England and Wales and percentage of county urban (1901-10)	$+.570 \pm .061$
pneumonia .....	$+.600 \pm .058$
respiratory diseases in 55 counties of "England and Wales and percentage of county urban (1901-10) ... ..	$+.627 \pm .055$
respiratory diseases in 42 counties of England and percentage of county urban (1891-1900)	$+.653 \pm .060$
Mean actual temperature in:	
55 counties of England and Wales and percentage of county urban (1901-10) ... ..	$+.119 \pm .090$
42 " " " " (1891-1900) ... ..	$-.153 \pm .102$
Death-rate from:	
bronchitis in 55 counties of England and Wales and mean annual temperature with percentage of county urban constant (1901-10) ... ..	$-.468 \pm .071$
pneumonia in 55 counties of England and Wales and mean annual temperature with percentage of county urban constant (1901-10) ... ..	$-.392 \pm .077$
respiratory diseases in 55 counties of England and Wales and mean annual temperature with percentage of county urban constant (1901-10) ... ..	$-.459 \pm .072$
respiratory diseases in 42 counties of England and mean annual temperature with percentage of county urban constant (1891-1900) ... ..	$-.334 \pm .092$

and pneumonia together or respiratory diseases for the period 1901-10 and (4) respiratory diseases for the period 1891-1900, respectively, are all negative. They are all approximately of the value  $-0.3$  and three, at least, appear to satisfy the criterion of statistical significance, the fourth and smallest—that between pneumonia and temperature—being not quite but approximately three times its probable error. These values would seem to indicate that there is an inverse relationship between the mortality from the diseases in the registration counties and their mean actual temperature. It is well known, however, that the amount of respiratory disease is influenced by the presence or absence of urban or industrial conditions and so variations in regard to these in the different counties might account, in some degree, for the apparent relationship found. In the supplement to the Registrar-General's report for 1901-10 there is given, however, for each registration county, the proportion of the county which is urban, taking London as 100. The correlation coefficients found between the mortality in the different counties from bronchitis, pneumonia and respiratory diseases, respectively, and the proportion of the county urban, were determined and were found to be approximately  $+0.6$ .



As the proportion of the county urban may be regarded as a very rough index of the intensity of industrial conditions therein, it has been possible, by the method of partial correlation, to determine the relationship between the mortality from the respiratory diseases and the mean annual temperature, some allowance having been made for environmental conditions. The partial correlation coefficients that result are still statistically significant and being negative indicate that there is a real inverse association between the mean annual temperature and the death-rates from the diseases, the higher mortality being found in the colder counties.

*Mortality from bronchitis and pneumonia in registration counties  
and rainfall.*

The correlation coefficients between the death-rates from bronchitis, pneumonia and respiratory diseases, respectively, in the registration counties and the corresponding rainfall are quite insignificant showing that there is no apparent relationship between the mortality from these diseases and the amount of rain that falls; thus confirming the conclusions that have been drawn from the results from the monthly and weekly data in the several towns investigated.

SUMMARY AND CONCLUSIONS.

In the preceding pages, an analysis has been made of the correlation coefficients found between the number of fatal cases from bronchitis, pneumonia and respiratory diseases, or the two summed together, in children under five years of age in London, Glasgow, Edinburgh, Aberdeen and Dundee, and the meteorological factors, mean and mean minimum temperature, mean relative humidity and mean rainfall, for a period of from 40 to 50 years; and between the death-rates from bronchitis, pneumonia and respiratory diseases amongst children at the same period of life in the registration counties of England and Wales in the decennia, 1901-10 and 1891-1900, and their mean annual temperature and mean annual rainfall and it seems warrantable to draw the following conclusions.

1. The meteorological factor, amongst those investigated, that seems to exercise the greatest influence in predisposing to an increased mortality from, and presumably an increased incidence of, bronchitis in children in the cities, is the prevailing temperature. In accordance with the average duration of the disease, the mortality is most intimately related to the mean temperature of the preceding week and is, on the average, as closely related to the temperature of two weeks before as it is to that of the corresponding week. The relationship is inverse, the lower the mean temperature the greater the fatality from bronchitis. As suggested by Dr Leonard Hill, the cold weather probably compels children to remain indoors in stuffy, overheated rooms where infection is intensified and health weakened by heat stagnation, a lowered metabolism and want of sunlight.

2. While there is a suggestion from some of the correlation coefficients found, that pneumonia in children may have some inverse relationship to mean temperature this association is not shown in the monthly or weekly data generally.

3. There is a definite inverse relationship between the mortality from the respiratory diseases, or bronchitis and pneumonia summed together, and the mean temperature; and, as occurs with bronchitis, the relationship to the mean temperature of the preceding week is closer than to that of the corresponding week.

4. The mortalities from bronchitis, pneumonia and respiratory diseases (bronchitis and pneumonia summed together), in children under five years in the registration counties of England and Wales, are inversely associated with the mean annual temperature in the corresponding counties. This association still persists after allowance is made for the proportion of each county which is urban, a proportion which has been taken as a rough index of industrial conditions therein.

5. The mortalities from bronchitis and pneumonia in children in the cities examined and in the registration counties are not influenced in any consistent manner or degree by the amount of rainfall.

6. While the coefficients of correlation between the corrected monthly deaths from bronchitis and respiratory diseases, respectively, and the corresponding mean monthly humidity in Glasgow seem to suggest the existence of a significant direct association between these variables, viz. the moister the atmosphere, the higher the death-rate from bronchitis; such a relationship is not definitely indicated in the coefficients found for the other cities. The magnitude of the coefficients for Glasgow is apparently determined, in some degree, by the periodicity in the mortality figures. It is probable, however, that the periodicity is not wholly responsible for the correlation found as the correlation coefficients between the mean temperature and the deviations in the same monthly data from the five-yearly moving average—the method adopted to eliminate the effect of the periodicity—are not very different from those found by using the actual figures. There would appear to be some evidence for the view that a high relative humidity, when associated with a low temperature, has some influence in predisposing to an increased mortality from the respiratory diseases.

## ANTHROPOMETRIC STUDIES OF GLASGOW SCHOOL CHILDREN.

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(With 2 Graphs.)

THE object of the present investigation was to determine the relation between the variations in hair and eye colours and variations of weight and stature; so that one might know what allowances, if any, would be necessary to make for variations of the proportion of children in different classes of hair and eye colour in different age groups and in the two sexes in the different schools under investigation with respect to the factors influencing bodily growth and nutrition. Incidentally, the analysis might reveal racial differentiation if such were present in this population.

The observations were made on children from three elementary schools under the Glasgow School Board by Miss Tully and Dr Weir. Of the 2801 children examined in this part of the study, there were 1362 males and 1439 females in age groups ranging from eight to thirteen.

The observed weights were taken correct to 0.5 lb.; the heights to 0.25 in. in indoor clothes without boots.

The different types of hair were classified in four categories; fair (F.), light brown (L.B.), dark brown (D.B.) and red (R.).

F. includes white, flaxen and golden yellow.

L.B.     ,,     all shades of brown except dark.

D.B.     ,,     dark brown and jet black.

R.        ,,     the various shades of red (light, bright and dark red).

The eye colours were grouped in five categories; light (L.), light medium (L.M.), medium (M.), dark medium (D.M.) and dark (D.).

L. includes light shades of grey.

L.M.     ,,     blue and bluish grey.

M.        ,,     pure grey, orange and mixed shades.

D.M.     ,,     brown and dark brown.

D.        ,,     black.

Table I shows the means and standard deviations of weights and heights for each age and sex group<sup>1</sup>. The standard deviation, which is a measure of the absolute variability of any quantity, is seen to increase with age in both sexes, and to be higher in females than in males in all age groups in the case

<sup>1</sup> Owing to the heavy expense of printing, it is not possible to reproduce the tabulations from which the statistical constants in the text tables were calculated; but anyone interested can consult the originals on application to the author.

of weights. The variability in heights is much smaller, and shows no tendency to increase with age or to differ in the sexes.

The coefficient of variation, a somewhat better comparative measure of the variability, which expresses the standard deviation as a percentage of the mean ( $\frac{\text{Standard Deviation} \times 100}{\text{Mean}}$ ) was then worked out (Table II). This table shows

the greater variability of females in comparison with males, and the variability in weights increasing with age; but when taken in relation to the probable errors, the differences observed do not attain to the conventional

Table I.

*Males.*

Age	Mean weight	Standard deviation	Mean height	Standard deviation	Total
8	50.85	5.46	46.25	2.64	236
9	55.44	6.82	47.77	2.68	232
10	60.55	7.54	49.96	2.66	246
11	65.02	7.46	51.67	2.82	241
12	68.40	8.52	52.71	2.96	204
13	74.99	10.67	54.71	3.32	203

*Females.*

8	49.48	5.88	46.00	2.41	261
9	53.91	7.00	47.85	2.76	278
10	57.39	7.57	49.57	2.78	246
11	61.29	8.09	51.13	2.66	238
12	69.43	11.43	53.58	2.93	228
13	78.48	12.76	56.00	3.29	188

Table II.

*Coefficients of Variation.*

	Weights		Heights	
	Males	Females	Males	Females
VIII	10.73 $\pm$ .337	11.88 $\pm$ .356	5.70 $\pm$ .178	5.25 $\pm$ .155
IX	12.29 $\pm$ .391	12.98 $\pm$ .377	5.61 $\pm$ .176	5.76 $\pm$ .165
X	12.45 $\pm$ .384	13.20 $\pm$ .408	5.32 $\pm$ .162	5.61 $\pm$ .171
XI	11.46 $\pm$ .357	13.20 $\pm$ .415	5.45 $\pm$ .168	5.20 $\pm$ .161
XII	12.45 $\pm$ .422	16.46 $\pm$ .534	5.62 $\pm$ .188	5.47 $\pm$ .173
XIII	14.22 $\pm$ .485	16.26 $\pm$ .580	6.06 $\pm$ .204	5.88 $\pm$ .205

standard of significance ( $3 \times$  probable errors) except in the instances of children of eleven and twelve years of age. The probable error of the difference between two uncorrelated magnitudes is equal to the square root of the sum of the squares of the probable errors of the quantities entering into the difference. Thus (*e.g.*) in children of eight years the difference between the coefficients of variation is  $(11.88 - 10.73) = 1.15$ .

The probable error of this difference is  $\pm \sqrt{.11357 + .12674} = \pm .490$ . The difference is thus  $1.15 \pm .490$ .

Similarly for the other age groups, the differences are in the case of weights as follows:

$$0.69 \pm .54; 0.75 \pm .56; 1.74 \pm .55; 4.01 \pm .68 \text{ and } 2.04 \pm .76.$$

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The observed differences are thus individually insignificant. The general tendency, however, to increase with age is so obvious, and the greater variability of females over males in weights is so constant, that they may well represent a real distinction although it cannot be statistically demonstrated having regard to the size of the probable errors.

The coefficients of variation in stature show no relation to age, and no constant difference between the sexes.

The correlations between height and weight are given in Table III. These, as might be expected, are all high; and there is no material difference in this respect between males and females, or at different ages.

The correlations between hair colour and eye colour were worked out by the product moment (Table IV) and contingency methods. The coefficients are

Table III.

*Correlation. Height—weight.*

Age	Males	Females
VIII	$\cdot 9277 \pm \cdot 006$	$\cdot 7644 \pm \cdot 017$
IX	$\cdot 7522 \pm \cdot 019$	$\cdot 7995 \pm \cdot 015$
X	$\cdot 6669 \pm \cdot 024$	$\cdot 8090 \pm \cdot 015$
XI	$\cdot 7349 \pm \cdot 020$	$\cdot 7704 \pm \cdot 018$
XII	$\cdot 7344 \pm \cdot 022$	$\cdot 7280 \pm \cdot 021$
XIII	$\cdot 7860 \pm \cdot 018$	$\cdot 7827 \pm \cdot 019$

Table IV.

*Correlation. Hair—eye colour.*

Age	Males	Females
VIII	$\cdot 3176 \pm \cdot 039$	$\cdot 1718 \pm \cdot 041$
IX	$\cdot 2341 \pm \cdot 042$	$\cdot 2928 \pm \cdot 037$
X	$\cdot 2455 \pm \cdot 040$	$\cdot 2144 \pm \cdot 041$
XI	$\cdot 3139 \pm \cdot 039$	$\cdot 2737 \pm \cdot 040$
XII	$\cdot 2469 \pm \cdot 044$	$\cdot 4274 \pm \cdot 037$
XIII	$\cdot 2202 \pm \cdot 045$	$\cdot 3647 \pm \cdot 043$

all positive; and in view of the comparatively low probable errors, may be taken as significant, although, having regard to the arbitrary assumptions involved, the actual values do not merit much attention<sup>1</sup>. The coefficients are of about the same order of magnitude as found by other writers by different methods. They show, as we should expect, that there is a predominance of the blonde and brunette types over those with the anomalous combinations of dark hair and light eyes and *vice versa*.

On the other hand, the lowness of the correlation indicates a tendency to homogeneity among the Glasgow school children. As pointed out by Tocher<sup>2</sup>, if there were two races, one of the blonde and one of the brunette type, present in a population in equal proportions, the correlation between hair and eye colours would approach unity. But the more this population in time and through intermarriage was thoroughly crossed, or the more nearly it came to

<sup>1</sup> It is wholly arbitrary to assume that the differences separating hair-colour or eye-colour groupings can be replaced by a series of quantities and equal class units.

<sup>2</sup> *Biometrika*, vol. VI. p. 130.

consist of members entirely of either class, the smaller would be the value of the correlation, and the nearer would it approach zero. None of the above coefficients are of a high order of magnitude, the mean coefficient for all age and sex groups being  $\cdot 2769$ , it is obvious that heterogeneity is not a predominant feature of this population.

Table V shows the correlations between weight and stature and hair and eye colours. The data were first of all treated by determining the product moment coefficients of correlation. The difficulties and risk of fallacy in the application of such a method are obvious, as a quantitative value for any one type of hair or eye colour cannot yet be given from the lack of chemical or other data on the subject; so that it is impossible to group hair and eye colours in such a manner as to indicate measurable increases in the amount of pigment contained therein.

Table V.  
*Correlations.*

Age	Weight		Height		Total
	Hair colour	Eye colour	Hair colour	Eye colour	
VIII	$-.0430 \pm .044$	$-.0750 \pm .044$	$.0154 \pm .044$	$.0559 \pm .044$	236
IX	$-.0881 \pm .044$	$-.0950 \pm .044$	$.0317 \pm .044$	$-.0393 \pm .044$	232
X	$-.1142 \pm .042$	$-.0054 \pm .043$	$.0760 \pm .043$	$-.0838 \pm .043$	246
XI	$-.0373 \pm .043$	$-.0630 \pm .043$	$.0990 \pm .043$	$-.0382 \pm .043$	241
XII	$-.0587 \pm .047$	$-.0216 \pm .047$	$-.0960 \pm .046$	$-.0815 \pm .047$	204
XIII	$-.1009 \pm .047$	$-.0568 \pm .047$	$.0152 \pm .047$	$-.1539 \pm .046$	203

<i>Females.</i>					
VIII	$-.0864 \pm .041$	$-.0004 \pm .042$	$-.1340 \pm .041$	$-.0262 \pm .042$	261
IX	$-.0251 \pm .040$	$-.0839 \pm .040$	$.0164 \pm .040$	$-.0215 \pm .040$	278
X	$-.0351 \pm .043$	$-.0433 \pm .043$	$-.0502 \pm .043$	$-.0296 \pm .043$	246
XI	$-.1680 \pm .042$	$-.0360 \pm .044$	$.0497 \pm .044$	$-.0603 \pm .044$	238
XII	$-.0587 \pm .045$	$.1220 \pm .044$	$.0741 \pm .044$	$-.0045 \pm .045$	228
XIII	$-.1925 \pm .047$	$-.0430 \pm .049$	$.0988 \pm .049$	$-.0446 \pm .049$	188

The tables, however, being so arranged as to indicate increasing pigmentation in each succeeding array, it was thought that this method would demonstrate linear regression, if such were present.

In the case of the relation between weight and hair colour there is evidence of some association amongst the older females. In males no such correlation is seen in any age group.

In both males and females in all age groups there is no demonstrable relationship between weight and eye colour or between stature and hair or eye colours.

It may be concluded, then, that there is no tendency for an increase (or decrease) in weight or stature to accompany an increase (or decrease) in the density of pigmentation in the hair or eyes. The significant coefficient for females in the correlation of weight with hair colour are so small that it is very doubtful if any importance attaches to these isolated observations.

The correlation ratios were then calculated for the relationship between weight and height with hair and eye colours (Table VI). The correlation ratio ( $\eta$ ) is a measure of the degree of association between two variables, and

is the best measure when the regression is non-linear. The observed ratios were then corrected by the method suggested by Pearson<sup>1</sup>:

$$\text{corrected } \eta^2 = \frac{\text{observed } \eta^2 - (K - 1)/N}{1 - (K - 2)/N},$$

where  $K$  = the number of arrays, and  $N$  the number of observations. The correction is made to allow for the influence of the number of arrays. The probable errors were calculated from these corrected values (in brackets). The values of  $\eta$  even when uncorrected for grouping are all very small; when

Table VI.  
*Correlation ratios.*

Sex	Age	Weight		Height	
		Eye colour	Hair colour	Eye colour	Hair colour
Males	VIII	.1448 (.0638 ± .044)	.1160 (.0274 ± .044)	.0765	.1152 (.0237 ± .044)
Females	VIII	.0914	.1371 (.0858 ± .041)	.0608	.1314 (.0763 ± .042)
Males	IX	.1313	.0909	.0777	.0277
Females	IX	.1284 (.0461 ± .040)	.1576 (.1189 ± .040)	.1718 (.1237 ± .040)	.1341 (.0851 ± .040)
Males	X	.0607	.1684 (.1277 ± .042)	.2451 (.2106 ± .041)	.1104
Females	X	.1267	.0510	.2050 (.1615 ± .042)	.0774
Males	XI	.1032	.0617	.0861	.1518 (.1034 ± .043)
Females	XI	.0824	.1249 (.0550 ± .044)	.1437 (.0624 ± .044)	.2294 (.2009 ± .042)
Males	XII	.0986	.1734 (.1246 ± .046)	.1105	.1936 (.1517 ± .046)
Females	XII	.1710 (.1089 ± .044)	.1039	.0958	.0687
Males	XIII	.0638	.2705 (.2428 ± .045)	.1614 (.0803 ± .047)	.2189 (.1829 ± .046)
Females	XIII	.1013	.2063 (.1640 ± .048)	.1226	.1832 (.1334 ± .048)

corrected, some become indeterminate, others are scarcely significant. In a few scattered cases the ratios are significant in relation to the probable errors. It was therefore thought advisable to calculate the mean weights and statures for each array of hair and eye colours; and then to determine if the differences of these from the mean values of the whole were significant. The means are given on Tables VII and VIII. As our criterion of significance we might either take three times the probable error of the difference of the mean value of any one array from the mean of the whole series, or  $2\sigma/\sqrt{N}$  (where  $\sigma$  = standard deviation of the whole series;  $N$  = the number of observations in any individual array). In this case we have chosen the latter. The mean of the series may be represented by a horizontal line on the graph, and on either side of this are plotted the values of  $\pm 2\sigma/\sqrt{N}$  for each type of hair and eye colour.

<sup>1</sup> *Biometrika*, vol. VIII. p. 254.

The areas so obtained represent the limits of the deviations on either side of the mean which might be expected to occur solely from the influence of random sampling. If the means of the arrays fall within this area, they cannot be safely said to differ significantly from the average. The results are illus-

Table VII.

*Males.*

Age	Average heights in relation to eye colour					Average heights in relation to hair colour			
	L.	L.M.	M.	D.M.	D.	F.	L.B.	D.B.	R.
VIII	45.96	46.05	46.35	46.58	46.22	45.69	46.29	46.61	43.50
IX	47.68	47.04	47.87	47.87	47.26	47.73	47.75	47.92	48.20
X	50.11	50.36	49.84	49.48	49.88	49.43	49.98	49.77	52.00
XI	52.44	51.44	51.67	51.72	51.50	52.71	51.47	52.56	51.00
XII	53.23	52.50	52.91	52.24	52.32	52.75	52.07	53.23	50.00
XIII	56.33	55.28	54.64	54.61	53.93	56.25	54.52	54.71	59.25

Age	Average weights in relation to eye colour					Average weights in relation to hair colour			
	L.	L.M.	M.	D.M.	D.	F.	L.B.	D.B.	R.
VIII	49.56	50.47	50.92	52.30	49.96	50.23	50.85	51.06	44.00
IX	57.12	55.62	55.13	56.04	53.74	55.53	55.30	56.17	59.40
X	59.53	61.11	60.35	60.48	60.47	55.92	60.81	59.96	65.00
XI	67.11	64.71	65.03	65.64	63.97	62.86	65.15	65.00	69.00
XII	66.68	69.15	68.86	67.30	68.80	67.63	68.45	70.06	61.75
XIII	75.00	76.12	74.98	74.77	73.52	80.88	74.02	75.35	92.75

Table VIII.

*Females.*

Age	Average heights in relation to eye colour					Average heights in relation to hair colour			
	L.	L.M.	M.	D.M.	D.	F.	L.B.	D.B.	R.
VIII	43.96	46.21	46.03	45.70	46.00	46.39	46.13	45.35	45.56
IX	48.65	48.20	47.36	48.45	47.90	47.07	47.98	47.34	49.33
X	50.89	49.73	48.97	49.69	50.18	50.31	49.52	49.63	49.11
XI	52.00	51.31	50.80	50.93	51.40	50.93	51.04	51.37	51.40
XII	54.00	53.34	53.42	54.09	53.55	53.26	53.49	53.78	54.50
XIII	57.08	56.26	55.78	55.55	56.34	53.83	56.09	56.41	55.50

Age	Average weights in relation to eye colour					Average weights in relation to hair colour			
	L.	L.M.	M.	D.M.	D.	F.	L.B.	D.B.	R.
VIII	48.75	50.44	49.93	48.42	49.47	49.94	49.80	47.70	50.44
IX	54.59	54.95	53.55	54.86	52.30	50.40	54.48	49.19	53.00
X	59.21	58.10	56.60	56.45	58.61	57.13	57.61	56.59	57.11
XI	62.71	61.61	60.67	61.07	61.90	57.93	61.45	62.37	62.80
XII	64.72	69.34	68.69	72.12	69.88	65.37	69.34	69.30	71.75
XIII	76.85	81.32	78.57	79.10	80.83	69.42	79.57	80.76	81.20

trated by Graphs I and II. (Space does not permit the reproduction of all the other graphs; but the salient features are noted below.)

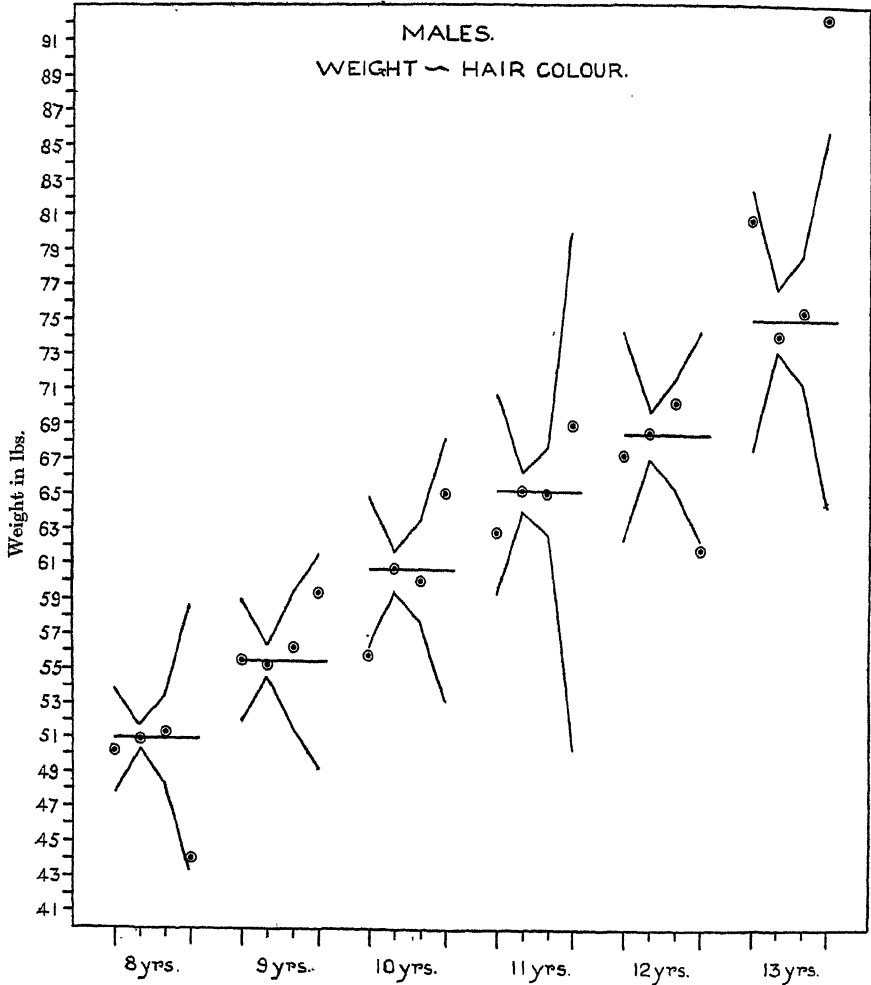
In Graph I (Males: Weight—Hair Colour) it is seen that only in three cases does the mean of any array differ sensibly from the average of the whole series. The weight of fair haired children at age 10 is just significantly below the mean. Red haired children at age 12 are below the mean weight; at age 13



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they are quite definitely above the mean. At all ages the weights of red-haired children deviate more widely from the general average than do those of any other type.

In none of the cases do the light brown or dark brown haired children differ significantly from the mean.



Graph I.

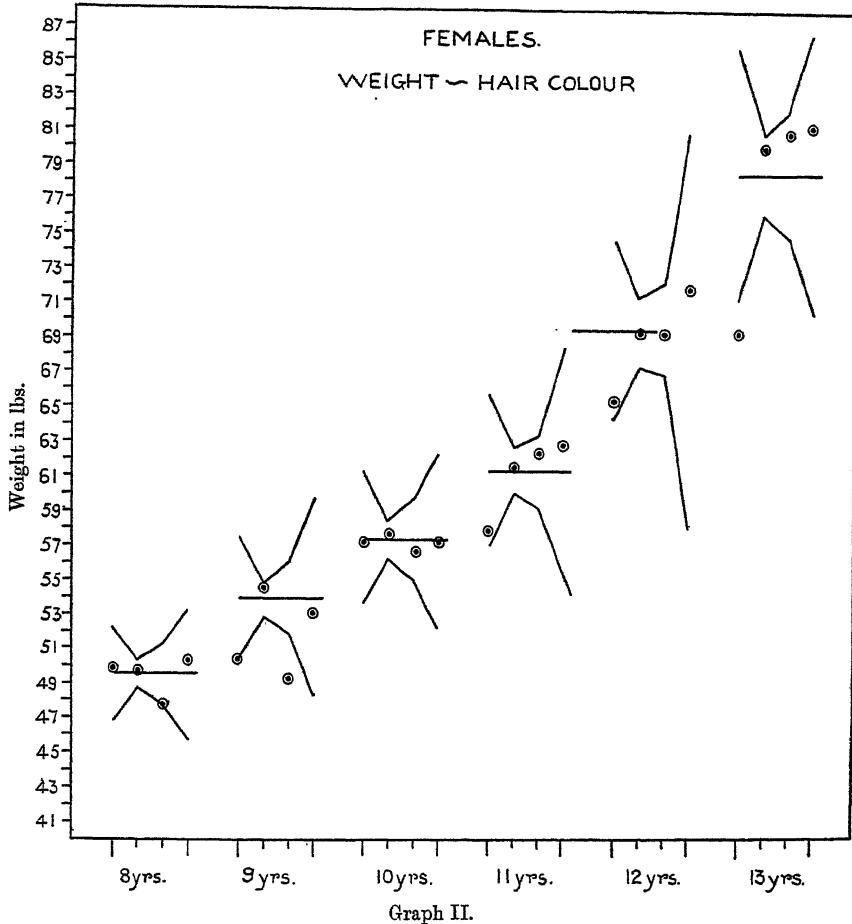
In the case of female children (Graph II), only two instances overstep the limits placed by  $2\sigma/\sqrt{N}$ ; dark-brown being below the mean at age 9; fair-haired being below the mean at age 12.

*Hair colour and stature.* Fair-haired females of age 13 are just below the mean; males of 12 with red hair are below, while those of 13 years are well above the average.

*Weight and eye colour.* In neither males nor females does the mean of any array differ significantly at any age from the mean of the whole series.

*Height—eye colour.* Light-eyed females at 8 years are below, at age 10 are above the mean for these ages. In males, none of the groups differ sensibly from the mean.

It would appear, then, that there is no important difference between the weights and statures of any group of hair or eye colour and the general average for the whole age group.



Tables IX and X have been drawn up to show the predominant types of hair and eye colours. They show the percentages of the different categories in the various age and sex groups.

There is a tendency for the percentage of fair-haired children to grow less as age advances, in the case of males. This does not appear to be so in females. The percentage of the light brown type tends also (though irregularly) to

diminish with age in both sexes. This diminution in fair and light brown is compensated for by the increase in the proportion of darker hair at the later ages. These results confirm the observation that hair tends to darken with age.

The proportion of red hair is extremely small in all age and sex groups, and there is no uniformity in the successive age groups. The number of observations on red-haired children in this material is too small for any reliable conclusions to be drawn from them.

The predominant type of hair in every group is light brown.

Table IX.

*Percentage of types of hair colour.*

Age	Sex	F.	L.B.	D.B.	R.
VIII	Males	5.51	86.02	7.63	0.84
	Females	6.89	73.18	16.48	3.45
IX	Males	6.47	86.21	5.17	2.15
	Females	5.39	75.54	16.91	2.16
X	Males	4.88	82.93	10.57	1.62
	Females	6.50	73.17	16.67	3.66
XI	Males	2.91	81.74	14.94	0.41
	Females	5.88	66.81	25.21	2.10
XII	Males	3.92	76.96	15.20	3.92
	Females	8.33	57.89	32.02	1.76
XIII	Males	3.94	77.34	16.75	1.97
	Females	6.38	61.17	27.13	5.32

Table X.

*Percentages of types of eye colours.*

Age	Sex	L.	L.M.	M.	D.M.	D.
VIII	Males	11.44	25.00	35.17	16.95	11.44
	Females	9.19	18.39	45.98	12.64	13.79
IX	Males	10.78	22.41	34.05	19.40	13.36
	Females	6.12	19.78	44.60	15.11	14.39
X	Males	7.72	30.49	32.52	16.26	13.01
	Females	7.72	25.61	38.21	17.07	11.39
XI	Males	7.47	18.67	43.57	16.18	14.11
	Females	10.08	22.69	43.28	11.34	12.61
XII	Males	10.78	12.75	48.04	16.18	12.25
	Females	10.96	17.98	42.10	14.48	14.48
XIII	Males	5.91	12.31	52.22	15.27	14.29
	Females	6.91	16.49	45.74	15.43	15.43

Table X shows that the predominant type of eye colour is that classed as medium. Group L. decreases in a somewhat irregular manner as age increases in the male series. In females there is no such definite trend. Light medium eyes show an increasing number up to the 10th year in both sexes, and then there is a gradual fall.

The proportion of medium eyes is quite definitely greater in older children in the male sex. Dark medium eyes show no definite relationship to age in either sex. Dark eyes are much more common in older than in the younger children. The increase with age, however, is very irregular.

The predominance of the shade of hair classed as L.B., and of eyes classed as M. has been shown by Tocher<sup>1</sup> to be positively correlated with density of population.

From this analysis, it would appear that there is no significant association between weight or stature and hair and eye colours.

The types of hair included under the class light brown are greatly in excess in all ages and sex groups; and there is significant defect in fair and red hair.

Medium eyes are in excess in both sexes at all ages.

The proportion of the lighter shades of hair and eye colours shows a fairly definite tendency to decrease with increasing age, more marked in the case of males than females.

Females are slightly more variable in weights than males; and this variability in weights has a tendency to increase with age.

There is no significant difference in the variability of stature between males and females.

In conclusion I should like to express my indebtedness to Prof. Noël Paton for giving me the opportunity of working out these results and to Dr M. Greenwood for his guidance and assistance in the prosecution of this study.

<sup>1</sup> *Biometrika*, vol. vi. p. 30.

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## THE PHYSIQUE OF GLASGOW SCHOOL CHILDREN (1921-22).

By ANNABEL M. T. TULLY, M.A.

*(From the Institute of Physiology, University of Glasgow.)*

In a study of the influence of environmental and other conditions on the growth and nutrition of the children of the poorer classes in the city of Glasgow, it seemed desirable to determine how far a homogeneous stock was being dealt with since there was some danger that racial variations might modify the results of the investigation.

For this purpose three typical schools attended by the class of children included in the above-mentioned investigation were selected and observations made upon colour of hair and eyes, weight, height and stem height.

The opportunity was taken of studying (1) the physique of these children as determined by weight, height and stem height, (2) how far recent illness tends to modify weight and height, and (3) how far the estimate of the condition of a child from its general appearance corresponds with the evidence afforded by weight and height.

Our purpose was not to re-investigate the relationship between social class and growth and nutrition, a matter which has already been very fully studied in this country and especially in Glasgow in the Public Schools, 1905-6, data from which have been analysed by Miss Elderton.

### *Method of Investigation.*

The method adopted was as follows:

Three investigators were employed and the work was allotted thus:

No. 1 weighed and measured the children.

No. 2 examined the colour of hair and eyes. It may be stated that the same individual carried out this part of the work in all the schools.

No. 3 obtained information relating to illness and classified the children according to their general appearance. This also was done by the same person in all the schools.

The information obtained was recorded on special cards drawn up as shown.

Further details as to method of weighing and classification are given later on in this report. Similar information in regard to the classification for colour of hair and eyes is given by Dr McKinlay in his paper on this subject (p. 176).

Our special thanks are due to Mr Clark of the Glasgow Education Authority who gave the most valuable help in selecting schools and in arranging for the

prosecution of the investigation, and to the Headmasters and Staffs of the Schools who gave every possible assistance in the carrying out of the work.

No compulsion was used in inducing the children to submit themselves to the examinations.

Name .....No.....  
 Age.....Sex .....  
 Weight.....lbs. Height.....ins. Stem length.....ins.  
 .....  
 Colour—Hair.....Eyes .....  
 Head Form—Length.....Breadth .....  
 General Appearance .....  
 Recent Illness .....  
 .....

---

M.R.C. CHILD LIFE (University of Glasgow).

The investigation was done under the Child Life Committee of the Medical Research Council at the instigation of Prof. Noël Paton. My grateful thanks are due to him for his assistance in preparing this paper.

The investigation was carried out during the closing month of 1921 and the opening months of 1922.

The schools, here designated A, B, C, are situated in three separate areas of the city, and although not originally selected for this reason are attended to some extent by the children of three different grades of workers.

School A is situated in an area in which there are few big factories or works, the district being mainly composed of tenement houses and this school is attended chiefly by children of boarding-house keepers, tradesmen and theatrical artistes, etc., with a sprinkling of a much poorer element coming from sunk basements in the neighbourhood. Both in clothes and in general health these children are superior to those in schools B and C.

School B is situated in a good working-class area, near shipyards and is attended by children of skilled engineers and other ship-yard workers and also of dockers and other unskilled workers of a distinctly poorer class.

School C is situated in a very poor locality. The housing in this district is extremely bad and the whole area is so built up that there are very few open spaces. The school is attended mainly by children of dockers and unskilled labourers—a large proportion being of Irish extraction.

The children were weighed and measured; their stem height was taken in the usual manner; a note was made of the general appearance of each child, whether healthy, fairly healthy, or not healthy, as also a record made of any illness during the past year.

The usual method of weighing and measuring in ordinary indoor garments

and without boots was adopted. In working out, the weights were taken to the nearest half pound and the heights to the nearest quarter inch.

Over 4000 children were examined, ranging in age from 4 to 14 years.

The ages are taken to the age last birthday so that the average age of each group is half a year more than the group figure, *i.e.* group 7 includes everything from 7-8 years and the mean age of the group is 7.5 years.

In the case of the children of 4 and 14 years the numbers were too small to be of value in this investigation and they were accordingly rejected.

*Number of children. 5-13 years.*

Number of boys, School A	366	Number of girls, School A	357
"      "      B	532	"      "      B	605
"      "      C	1014	"      "      C	1121
"      "      A, B, C	1912	"      "      A, B, C	2083

## RESULTS.

### *General Physique.*

Tables I and II give the arithmetical average weight, height and stem height of boys and girls at each age in each of the three schools. Table III gives the average weight and height at each age over the three schools combined.

The stem height is given along with the total height (Table II).

Table IV shows the percentage relation of stem height to full height at each age for the three schools combined. As is already known, this percentage falls steadily as age advances.

The figures in brackets in all tables indicate the number of children weighed and measured at each age.

Table I.

*Average weight in lbs.—Individual Schools.*

(a) *Boys.*

Age	A	B	C
5	40.70 (32)	41.25 (36)	38.45 (54)
6	44.33 (35)	44.36 (65)	43.67 (82)
7	48.07 (38)	46.97 (65)	47.56 (133)
8	52.30 (43)	51.97 (73)	49.66 (121)
9	55.62 (29)	56.57 (72)	54.76 (140)
10	64.11 (36)	58.52 (81)	61.00 (126)
11	67.84 (58)	63.87 (61)	64.27 (123)
12	73.13 (43)	65.58 (38)	67.79 (125)
13	80.47 (52)	68.13 (41)	74.60 (110)

(b) *Girls.*

Age	A	B	C
5	39.18 (30)	40.10 (46)	37.04 (80)
6	42.77 (38)	43.29 (88)	41.31 (106)
7	46.38 (32)	45.84 (70)	44.30 (147)
8	50.11 (42)	50.72 (81)	48.48 (140)
9	55.02 (43)	55.93 (83)	52.44 (154)
10	59.81 (40)	57.64 (75)	56.42 (130)
11	64.10 (41)	60.25 (75)	60.71 (125)
12	74.04 (37)	66.40 (63)	68.64 (127)
13	82.86 (54)	71.33 (24)	78.90 (112)

Table II.

*Average height and stem height in ins.—Individual schools.*

## (a) Boys.

Age	A		*	B		*	C		*
	Total height	Stem height		Total height	Stem height		Total height	Stem height	
5	41.99	23.33	(32)	40.78	22.95	(36)	40.32	22.28	(54)
6	44.06	23.81	(35)	42.75	23.42	(65)	42.39	23.31	(82)
7	46.03	24.64	(38)	44.48	24.58	(65)	44.34	24.02	(133)
8	47.85	25.32	(43)	46.67	25.59	(73)	45.38	24.33	(121)
9	48.89	25.80	(29)	48.20	26.19	(72)	47.08	25.03	(140)
10	51.77	26.72	(36)	49.77	26.59	(81)	49.53	25.85	(126)
11	52.84	27.24	(58)	51.80	27.76	(61)	50.89	26.42	(123)
12	54.53	28.16	(43)	52.65	27.73	(38)	52.11	26.68	(125)
13	56.88	29.37	(52)	53.93	28.21	(41)	53.94	27.64	(110)

## (b) Girls.

5	41.48	23.17	(30)	40.50	23.10	(46)	39.89	22.01	(80)
6	43.16	23.47	(38)	42.04	23.66	(88)	41.11	23.01	(106)
7	45.97	24.23	(32)	44.08	24.08	(70)	43.30	23.62	(147)
8	47.01	24.98	(42)	46.28	25.23	(81)	45.53	23.95	(140)
9	49.45	25.53	(43)	48.51	25.90	(83)	47.14	24.96	(154)
10	50.95	26.21	(40)	50.01	26.72	(75)	48.93	25.53	(130)
11	52.29	27.10	(41)	51.59	27.48	(75)	50.53	26.25	(125)
12	55.27	28.52	(37)	53.93	28.46	(63)	52.98	27.32	(127)
13	57.78	30.29	(54)	54.74	28.99	(24)	55.23	28.35	(112)

\* Indicates number of children whose stem height is stated. Rachitic children discounted in stem height (Table II, (a) and (b)).

Table III.

*Average weight in lbs. and height in ins.—Three schools combined.*

## (a) Boys.

Age	Weight	No. of observations	Height	No. of observations
5	39.9	(122)	40.9	(122)
6	44.0	(182)	42.8	(182)
7	47.5	(236)	44.7	(236)
8	50.9	(237)	46.2	(237)
9	55.4	(241)	47.6	(241)
10	60.6	(243)	49.9	(243)
11	65.0	(242)	51.6	(242)
12	68.5	(206)	52.7	(206)
13	74.8	(203)	54.7	(203)

Total number of boys=1912.

## (b) Girls.

5	38.4	(156)	40.4	(156)
6	42.1	(232)	42.0	(232)
7	45.0	(249)	43.9	(249)
8	49.4	(263)	46.0	(263)
9	53.9	(280)	47.9	(280)
10	57.4	(245)	49.6	(245)
11	61.2	(241)	51.2	(241)
12	68.9	(227)	53.6	(227)
13	79.1	(190)	55.9	(190)

Total number of girls=2083.



Table IV.

*Average stem height.—Three schools combined and per cent. relation stem height to full height\*.*

Age	Boys		Girls	
	Stem height in ins.	% Relation stem height to full height	Stem height in ins.	% Relation stem height to full height
5	22.76	55.49	22.56	55.54
6	23.45	54.60	23.34	55.53
7	24.23	54.05	23.83	53.90
8	24.92	53.52	24.52	53.19
9	25.49	53.24	25.33	52.77
10	26.23	52.50	26.00	52.37
11	26.96	52.22	26.78	52.35
12	27.18	51.53	27.83	51.84
13	28.21	51.15	28.98	51.56

Total number of boys = 1841. Total number of girls = 2035.

\* Not including rachitic children.

These tables show (1) that the height of the children corresponds with the economic position of the parents; (2) that in weight there is not the same clear distinction, (3) that it is after about nine years of age that the weight of the children in the better class school (A) is generally most markedly superior to that in the other two (B and C), and (4) that in boys after nine and in girls after eleven the children attending school C tend to increase in weight more than those in school B.

Table V.

*Average weight in lbs.—Comparison.*

## (a) Boys.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Age	Present investi- gation	British Association <i>All classes</i>	British Association Artisan class	Glasgow Kay	Glasgow Mackenzie	English children A. Greenwood	Dundee
5	39.87	39.9	40.9	—	38.6	38.7	37.50
6	44.04	44.4	44.6	39.19	41.8	42.2	41.75
7	47.48	49.7	50.7	46.27	45.3	46.4	45.50
8	50.85	54.9	54.3	49.06	49.3	52.0	49.50
9	55.40	60.4	58.3	51.91	53.6	55.9	57.50
10	60.63	67.5	64.0	57.41	58.3	60.4	59.87
11	65.03	72.6	69.0	60.74	63.1	66.4	62.75
12	68.50	76.7	73.0	65.33	68.1	72.7	68.62
13	74.80	82.6	79.0	74.41	73.5	77.4	74.25
No. examined	1912	10200	3070	351	34922	363928	505

## (b) Girls.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Age	Present investi- gation	British Association <i>All classes</i>	British Association Artisan class	Glasgow Kay	Glasgow Mackenzie	English children A. Greenwood	Dundee
5	38.35	39.2	40.3	—	37.8	37.7	37.00
6	42.05	41.7	43.1	37.84	40.6	41.2	41.00
7	45.00	47.5	46.2	43.72	43.9	44.9	45.62
8	49.43	52.1	51.8	45.66	47.5	49.6	47.25
9	53.87	55.5	55.2	51.86	51.9	54.1	55.50
10	57.35	62.0	60.5	54.87	56.1	58.7	58.25
11	61.17	68.1	66.8	62.28	61.1	65.5	64.50
12	68.90	76.4	74.9	65.55	67.2	73.9	67.50
13	79.07	87.2	84.9	75.78	75.1	80.4	75.00
No. examined	2083	2930	1523	356	34130	347125	517

Cols. (1), (2), (3), (6) and (7) weighed with indoor clothing. Col. (4) weighed without clothing. Col. (5) weighed with clothing.

Tables V and VI compare the weight and height of the children in the present investigation with the statistics for weight and height given by the British Association—Anthropometric Committee, 1883, all classes and artisan class, with Dr Kay's Tables of Height and Weight, etc. of Glasgow School Children, 1904, with "The Report on the Physical Conditions of Children attending the Public Schools of the School-Board of Glasgow" (Mackenzie), 1905-1907, with Mr Arthur Greenwood's Report on English School Children 1908-11; and with the Report of Dundee Social Union (1904-5).

Table VI.

*Average height in inches\*.—Comparison.*

## (a) Boys.

Age	Present investigation	British Association <i>All classes</i>	British Association Artisan class	Glasgow Kay	Glasgow Mackenzie	English children A. Greenwood	Dundee
5	40.90	41.03	39.72	—	40.0	40.7	39.37
6	42.84	44.00	41.90	41.63	41.9	42.8	41.78
7	44.65	45.97	44.60	45.40	43.7	45.0	44.19
8	46.22	47.05	46.46	47.27	45.8	47.4	46.06
9	47.63	49.70	48.88	49.34	47.7	49.2	48.82
10	49.94	51.84	50.72	50.59	49.6	50.8	49.90
11	51.58	53.50	52.68	52.15	51.3	53.0	51.38
12	52.72	54.99	53.72	53.27	53.0	54.9	52.90
13	54.70	56.91	55.81	55.67	54.6	56.1	54.53
No. examined	1912	12617	7863	351	34922	367043	508

## (b) Girls.

5	40.38	40.55	39.77	—	39.9	40.4	39.58
6	41.95	42.88	41.84	41.97	41.6	42.5	41.34
7	43.86	44.45	43.56	44.78	43.4	44.8	44.19
8	46.00	46.60	45.55	46.64	45.2	46.9	45.47
9	47.90	48.73	47.36	48.56	47.2	48.7	48.23
10	49.59	51.05	48.96	49.52	49.0	50.6	49.41
11	51.16	53.10	51.54	51.80	50.8	53.0	52.56
12	53.62	55.66	53.98	54.11	52.9	55.5	53.25
13	55.89	57.77	56.22	55.86	55.1	56.8	55.32
No. examined	2083	2802	1544	356	34130	352952	515

\* All measurements are without boots.

The ratio  $\frac{\text{weight in lbs.}}{\text{height in inches}}$  was calculated but it is at best an unreliable index of nutrition since a child backward in growth and under-nourished may yield the same quotient as one of normal stature and nutrition.

The only reliable method of using this index is to determine its standard for the normal child of each age and to compare the quotient in the case of each child with the standard quotient for its age.

Till we have more definite knowledge of what is to be considered the "normal" height and weight for each age this procedure is impossible.

1. *Children with Rachitic Deformities.*

Among the children studied a certain number showed marked rachitic deformities, curving of the leg bones, cranial bossing and pigeon chests.

# 192      *Physique of Glasgow School Children (1921-22)*

There were 71 boys and 48 girls distributed among the schools as follows:

	Boys		Girls	
	Total	Per cent.	Total	Per cent.
School A	2	0.55	0	0
" B	13	2.44	16	2.64
" C	56	5.52	32	2.85

The following Table (VII), shows the average weight, height and stem of the rachitic boys and girls at each age in the three schools combined.

Table VII.

*Rachitic children.—Three schools combined.*

Age	Boys				Girls			
	Weight	Height	Stem height	No. of observations	Weight	Height	Stem height	No. of observations
5	35.00	37.25	21.69	(4)	35.06	36.44	21.57	(9)
6	41.40	40.85	22.80	(10)	40.33	40.42	23.04	(12)
7	44.15	41.56	23.40	(13)	41.90	39.85	23.33	(10)
8	45.11	42.11	23.94	(18)	44.00	41.58	24.00	(6)
9	48.07	43.68	24.63	(14)	46.67	43.33	23.08	(6)
10	57.75	49.00	25.75	(4)	43.50	42.88	23.38	(2)
11	60.50	46.63	25.38	(2)	78.50	51.50	28.50	(1)
12	61.50	46.50	25.50	(1)	52.00	47.50	26.25	(1)
13	60.20	47.70	26.45	(5)	85.50	53.25	30.50	(1)

Total number of boys=71. Total number of girls=48.

The deficiency in weight in these children who have suffered from rickets is large. The apparent exceptions in girls of 11 and 13, are not significant as there was only one individual in each group.

The comparatively small proportion of children with rachitic deformities is interesting in face of the very great prevalence of the disease in an acute form in children of the same community at an early age. It indicates either the high mortality among infants who develop the condition or the extent to which recovery from the disease takes place.

The proportion of stem height to total height is much greater in these rachitic cases as is shown by the following table (VIII).

Table VIII.

*Stem height to total height.—Per cent.*

Age	Boys		Girls	
	Non-rachitic	Rachitic	Non-rachitic	Rachitic
5	55.5	58.2	55.5	59.2
6	54.6	55.8	55.5	57.0
7	54.1	56.3	53.9	58.5
8	53.5	56.9	53.2	57.7
9	53.2	56.4	52.8	53.3
10	52.5	52.6	52.4	54.5
11	52.2	54.4	52.4	55.3
12	51.5	54.8	51.8	55.3
13	51.2	55.5	51.6	57.3

The age at which there is the largest number of rachitic children, viz. boys 11, was selected and the following correlations worked out on the rachitic and non-rachitic children.

Table IX.

Correlation	Non-rachitic (219)	Rachitic (18)
Weight to height	$+ \cdot 695 \pm \cdot 024$	$+ \cdot 712 \pm \cdot 078$
Weight to stem height	$+ \cdot 592 \pm \cdot 030$	$+ \cdot 801 \pm \cdot 057$
Height to stem height	$+ \cdot 583 \pm \cdot 030$	$+ \cdot 759 \pm \cdot 067$

Thanks are due to Dr McKinlay for working out the correlations on the non-rachitic children.

## 2. *Effect of recent illness.*

To ascertain whether recent illness tended to affect weight and height information was sought regarding any illness during the past year sufficiently serious to prevent attendance at school.

In Tables X and XI the children who had been ill during the year are tabulated with those who had had no illness. It will be seen from these tables that the results of this combination show no significant difference; at one age children who have been ill being taller and heavier than those who have not been ill and at the following age *vice versa*.

Many of the "illnesses" were of a very minor nature and judging by outward appearances some of the children who had been ill looked more robust than those who had not been off school. Recent illness except in those

Table X.

*Illness and no illness. Weight in lbs.*

<i>Boys.</i>							
Age	A		B		C		
	(a)*	(b)†	(a)	(b)	(a)	(b)	
5	39.3 (18)	42.6 (14)	44.8 (3)	40.9 (33)	38.0 (12)	38.6 (42)	
6	44.2 (11)	44.4 (24)	44.7 (26)	44.2 (39)	44.3 (29)	43.3 (53)	
7	48.4 (11)	47.9 (27)	47.9 (25)	46.4 (40)	47.8 (42)	47.5 (91)	
8	52.9 (10)	52.1 (33)	53.3 (25)	51.2 (48)	50.3 (37)	49.4 (84)	
9	58.2 (9)	54.5 (20)	56.9 (28)	56.4 (44)	55.1 (44)	54.6 (96)	
10	65.8 (15)	63.0 (21)	57.8 (29)	59.0 (52)	61.2 (40)	60.9 (86)	
11	66.9 (22)	68.4 (36)	64.2 (24)	63.7 (37)	65.6 (35)	63.8 (88)	
12	71.6 (11)	72.7 (32)	66.3 (16)	65.6 (22)	66.5 (42)	68.5 (83)	
13	78.3 (19)	81.7 (33)	68.6 (17)	67.8 (24)	72.2 (32)	75.6 (78)	
Number	126	240	193	339	313	701	
<i>Girls.</i>							
5	38.4 (7)	39.4 (23)	41.0 (15)	39.7 (31)	36.6 (12)	37.1 (68)	
6	43.3 (11)	42.6 (27)	42.6 (39)	43.8 (49)	41.0 (45)	40.5 (61)	
7	45.9 (8)	46.5 (24)	45.4 (28)	46.1 (42)	44.4 (52)	44.3 (95)	
8	49.1 (16)	50.7 (26)	50.5 (37)	50.9 (44)	48.8 (49)	48.3 (91)	
9	57.0 (17)	53.7 (26)	54.6 (44)	57.4 (39)	52.4 (53)	52.4 (101)	
10	62.2 (12)	58.8 (28)	57.3 (31)	57.9 (44)	56.4 (47)	56.4 (83)	
11	64.4 (12)	64.2 (29)	59.7 (23)	60.5 (52)	59.3 (41)	61.5 (84)	
12	76.8 (11)	74.1 (26)	65.4 (26)	67.1 (37)	68.2 (39)	68.8 (88)	
13	82.7 (22)	83.0 (32)	70.2 (13)	72.7 (11)	77.6 (40)	79.6 (72)	
Number	116	241	253	349	378	743	

\* (a) = Been ill.

† (b) = No illness.

cases where there were complications did not seem materially to affect the physique of the child. It may even be that illness, if not too severe, entailing rest and special care at home, may in some cases be beneficial to the health of the child.

Table XI.  
*Illness and no illness. Height in inches.*

Age	A		B		C	
	(a)*	(b)†	(a)*	(b)†	(a)*	(b)†
5	41.2 (18)	43.0 (14)	42.5 (3)	40.6 (33)	41.2 (12)	40.1 (42)
6	43.3 (11)	44.4 (24)	43.0 (26)	42.6 (39)	43.0 (29)	42.3 (53)
7	46.1 (11)	46.0 (27)	45.3 (25)	44.0 (40)	44.7 (42)	44.3 (91)
8	48.4 (10)	47.7 (33)	47.0 (25)	46.5 (48)	45.8 (37)	45.3 (84)
9	49.4 (9)	48.6 (20)	48.5 (28)	48.0 (44)	47.1 (44)	47.1 (96)
10	52.5 (15)	51.2 (21)	50.0 (29)	49.7 (52)	49.8 (40)	49.5 (86)
11	53.0 (22)	52.8 (36)	54.7 (24)	51.3 (37)	51.4 (35)	50.7 (88)
12	54.2 (11)	54.7 (32)	52.9 (16)	52.5 (22)	51.7 (42)	52.2 (83)
13	56.6 (19)	57.1 (33)	54.5 (17)	53.5 (24)	53.6 (32)	54.1 (78)
Number	126	240	193	339	313	701

*Girls.*

5	41.5 (7)	41.5 (23)	41.0 (15)	40.2 (31)	41.1 (12)	39.7 (68)
6	41.4 (11)	43.9 (27)	42.1 (39)	42.0 (49)	42.3 (45)	40.8 (61)
7	46.1 (8)	45.9 (24)	44.1 (28)	44.1 (42)	43.7 (52)	43.2 (95)
8	46.6 (16)	47.3 (26)	46.6 (37)	46.0 (44)	45.5 (49)	45.6 (91)
9	50.8 (17)	48.6 (26)	48.3 (44)	48.7 (39)	47.3 (53)	47.1 (101)
10	51.8 (12)	50.6 (28)	49.4 (31)	50.5 (44)	49.2 (47)	48.7 (83)
11	52.7 (12)	52.2 (29)	51.1 (23)	51.8 (52)	50.4 (41)	50.6 (84)
12	55.2 (11)	55.3 (26)	53.4 (26)	54.3 (37)	52.9 (39)	53.0 (88)
13	58.2 (22)	57.5 (32)	53.9 (13)	55.7 (11)	55.0 (40)	55.4 (72)
Number	117	241	256	349	378	743

\* (a) = Been ill.

† (b) = No illness.

The uniformity in the percentage of those who have been ill and those who have not been ill in the different schools is interesting.

	Boys		Girls	
	Been ill	No illness	Been ill	No illness
School A	% 34.4	% 65.6	% 32.5	% 67.5
„ B	36.3	63.7	42.3	57.7
„ C	30.9	69.1	33.7	66.3

3. *Correlation of classification of children by general appearance and by weighing and measuring.*

In what is known as the Dunfermline system an attempt is made to classify the state of nutrition of children by observation of the general appearance—the condition of skin, muscular tone and development, vigour, facial expression, voice and interest are the chief factors considered in forming this estimate. The children are classified into four groups as:

- (1) Excellent, *i.e.* nutrition of a healthy child of “good social standing.”
- (2) Good, *i.e.* nutrition just falls short of this “excellent” standard (1).

- (3) Requiring Supervision, *i.e.* nutrition on the border-line of serious impairment.  
 (4) Requiring medical treatment, *i.e.* nutrition seriously impaired.

The percentage number of children in each group is thus calculated.

An adaptation of this method, as described in the following paragraph, was used by us.

Before the weight and height of each child were known, its general appearance was examined by an experienced observer and recorded on the card as

- (a) Markedly healthy.  
 (b) Fairly healthy.  
 (c) Not healthy.

In thus classifying the children attention was paid to (a) colour of skin, (b) general vivacity and behaviour of child when not conscious of observation, and (c) the general build of the child. With regard to classification according to build, it was found that many children were naturally slight and wiry. Of this class a particularly noticeable feature in the case of the girls in school A, was that although they were vivacious and had had no illness, they appeared to be below the normal standard both in weight and height.

On the other hand, there were children who seemed to be above the

Table XII.

*Three schools combined. General appearance\*. Weight in lbs.*

(a) Boys.			
Age	(a)	(b)	(c)
5	40.9 (48)	39.9 (29)	37.0 (12)
6	44.4 (72)	43.3 (30)	42.2 (14)
7	48.3 (82)	46.2 (36)	46.3 (40)
8	52.6 (73)	49.1 (44)	48.4 (48)
9	57.4 (74)	53.1 (50)	52.3 (36)
10	62.1 (74)	59.2 (46)	58.5 (39)
11	67.9 (82)	62.4 (39)	60.9 (40)
12	74.0 (64)	64.9 (42)	63.6 (31)
13	80.9 (71)	70.9 (39)	68.6 (25)

(b) Girls.			
Age	(a)	(b)	(c)
5	39.0 (75)	37.8 (30)	35.6 (17)
6	43.1 (86)	40.9 (25)	40.0 (26)
7	45.9 (82)	45.1 (39)	43.4 (40)
8	51.5 (88)	46.6 (42)	45.7 (31)
9	55.7 (95)	53.1 (42)	48.5 (29)
10	59.3 (92)	55.4 (42)	51.9 (21)
11	64.9 (96)	58.1 (45)	55.1 (24)
12	71.4 (96)	67.5 (34)	62.4 (21)
13	84.0 (78)	71.1 (26)	71.4 (11)

(a)=Markedly healthy. (b)=Fairly healthy. (c)=Not healthy.

\* To test the homogeneity of the material, the correlation ratio ( $\eta$ ) of General Appearance upon Age was computed separately for each school, for boys the values were  $0.13 \pm .04$ ,  $0.07 \pm .04$ ,  $0.19 \pm .03$ ; for girls,  $0.20 \pm .04$ ,  $0.13 \pm .04$ ,  $0.18 \pm .02$ . The values were corrected for grouping and the probable errors are those for uncorrelated material. It will be seen that there is no consistent difference.

standard in weight and height who had grey pasty complexions and their faces exhibited impetiginous or other superficial suppurative rashes. Such children were seen among the boys in school C and could not under any circumstances be said to be healthy.

In spite of the fact that the former were classed as healthy and the latter as not healthy, there is on the whole, with reference to build, a distinct difference in the various groups into which they were divided.

In the following tables (XII and XIII) the recorded appearance of the children without history of recent illness is compared with the weight and height at each age.

They show clearly that the general appearance of a child gives a fair indication of its growth and nutrition.

Table XIII.

*Three schools combined. General appearance. Height in ins.*

*Boys.*

Age	(a)	(b)	(c)
5	41.2 (48)	40.7 (29)	39.0 (12)
6	43.0 (72)	42.5 (30)	41.5 (14)
7	45.0 (82)	44.1 (36)	43.9 (40)
8	47.2 (73)	45.4 (44)	44.8 (48)
9	48.4 (74)	47.1 (50)	46.3 (36)
10	50.4 (74)	49.4 (46)	48.9 (39)
11	52.2 (82)	50.8 (39)	49.9 (40)
12	54.0 (64)	52.2 (42)	51.5 (31)
13	56.0 (71)	53.6 (39)	52.6 (25)

*Girls.*

5	40.5 (75)	40.0 (30)	38.8 (17)
6	42.7 (86)	41.8 (25)	41.2 (26)
7	44.2 (82)	43.6 (39)	43.5 (40)
8	46.6 (88)	45.5 (42)	44.8 (31)
9	48.3 (95)	47.6 (42)	45.8 (29)
10	50.1 (92)	49.3 (42)	47.8 (21)
11	52.1 (96)	50.6 (45)	49.3 (24)
12	54.4 (96)	53.1 (34)	51.9 (21)
13	56.7 (78)	54.7 (26)	54.2 (11)

(a) = Markedly healthy.

(b) = Fairly healthy.

(c) = Not healthy.

The proportion of children classified in these three groups in the three schools is of interest, showing on the whole the greater proportion of the "markedly healthy" in school A and the distinctly higher proportion of those with a less healthy appearance in the poorer class school.

Percentage of children classed from their appearance (a) markedly healthy, (b) fairly healthy and (c) not healthy.

	Boys			Girls		
	A	B	C	A	B	C
	%	%	%	%	%	%
(a)	60.8	55.8	43.5	63.5	66.5	54.2
(b)	23.8	32.1	27.0	22.8	24.3	24.9
(c)	15.4	12.1	29.5	13.7	9.2	20.9

The following table (XIV) shows the average weights and heights in the three groups for all children between 5 and 13. Here again with the larger number a difference is perceptible in the various groups.

Table XIV.  
*General appearance. Age 5-13.*

	(a)	(b)	(c)
Boys. Weight in lbs.	59.12 (640)	55.15 (355)	54.35 (285)
Girls. „	57.51 (788)	52.89 (325)	48.71 (220)
Boys. Height in ins.	48.81 (640)	47.70 (355)	47.14 (285)
Girls. „	48.57 (788)	47.48 (325)	45.72 (220)

#### CONCLUSIONS.

1. Our results confirm the conclusion of Miss Elderton and show that in 1921-22 the children of what may be called the Artisan Class in Glasgow represented by School A, are, in weight and height, up to the standard of the Anthropometric Committee for the Artisan Class of the country generally.

2. On an average the children of the poorer labouring classes represented by School C are somewhat below this standard especially in height.

3. Children who have suffered from rickets and recovered with deformities are shorter and lighter than those who show no sign of the disease. The small percentage of children with rachitic deformity shows either (1) that the mortality of rachitic children is high, or (2) that the deformities may be largely recovered from.

4. There is no indication that the ordinary ailments of childhood involve any serious retardation of growth and nutrition.

5. The classification of children by their general appearance in the hands of an experienced observer gives a good indication of the state of their growth as indicated by the height and their nutrition as indicated by their weight.

I desire to thank Dr M. Greenwood and Miss C. M. Thompson for their advice and assistance in the preparation of this paper.

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## FURTHER INVESTIGATIONS INTO THE BIOLOGICAL CHARACTERISTICS OF *B. ENTERITIDIS* (*AERTRYCKE*).

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WE recently reported in this *Journal* (Topley and Ayrton, 1924 *b*) the results obtained by administering cultures of *B. aertrycke* to mice, *per os*, and studying the subsequent excretion of this organism in the faeces. From these results we concluded that there was a marked correlation between the antigenic structure of a given strain of *B. aertrycke*, as revealed by agglutination, and the capacity of that strain to give rise to persistent faecal excretion. In a later report (Topley and Ayrton, 1924 *c*) we studied certain other characters of this bacterial species, which vary discontinuously, and concluded that there was definite evidence of linkage between certain pairs of attributes, so that both members of a pair were either present or absent in any one strain.

Between the date when the proofs of these reports were finally corrected and the actual date of publication, we obtained results which showed us that certain of our conclusions were rendered invalid by an unsuspected source of error in our technique. By the courtesy of the Editor, we were enabled to attach to each report an addendum, entering a caveat against the acceptance of our conclusions as there stated.

The present report deals with certain serological relations, which have been revealed by a re-investigation of the points at issue, and with the results obtained in a repetition of those of our earlier experiments which had been rendered meaningless by the error in question.

We have been largely concerned with the reaction of our bacterial strains to the type and group agglutinins, whose existence has been demonstrated by Andrewes (1922). Reference to the reports in question will show that a notably large proportion of all the strains of *B. aertrycke* which we examined agglutinated both with a type and with a group antiserum. We were led to the conclusion that such a reaction indicated the presence of both type and group antigen in the individual bacilli, rather than the presence through variation, of both type and group bacilli in the same culture; though the occurrence of such mixed cultures was, of course, recognised to be a common occurrence under certain circumstances. The argument involved the tacit assumption that the antisera employed were revealing the presence of type and group antigens, and of these alone. This assumption was false.

<sup>1</sup> A Report to the Medical Research Council.

The effect of the time and temperature of incubation of cultures of *B. aertrycke*, on their subsequent agglutinability, has been referred to in earlier reports; and the tendency for cultures, when incubated for a long time at body temperature, to agglutinate with both group and type antisera has been emphasised. The mixed agglutination in such cases was, however, regarded as evidence of bacterial variation, taking place during the later phase of growth and multiplication.

In the course of a more recent experiment, in which numerous strains of *B. aertrycke* were tested by agglutination, we noted that incubation, even at 22° C. for a period of 24 hours or more, or incubation at 37° C. for so short a time as 16 hours, would result in a very high proportion of strains giving mixed agglutination. We noted also that, when a batch of tests showed many instances of mixed agglutination, strains reacting with the type serum alone were usually absent, while strains reacting with group serum alone might be present in considerable numbers. During the course of an experiment, in which the faeces from a large number of mice were examined each day, a high frequency of type strains, and an entire absence of mixed strains, would suddenly be replaced by a high frequency of mixed strains, and an entire absence of type strains, the relative frequency of the group strains being unaffected. Reference to the records showed that the mixed agglutination results occurred particularly with those cultures which were put up on a Saturday, and which were incubated at 22° C. until the Monday morning, or, in some instances, until the later hours of the Sunday. These observations led us to test a considerable series of strains with regard to the effect of variation in the time and temperature of incubation on the subsequent agglutinability of the cultures.

#### AGGLUTINATION EXPERIMENTS.

The method adopted was as follows. Plates were inoculated with a given strain of *B. aertrycke*, and were incubated over night. Next day several well separated colonies were selected on each plate, a portion of each colony was removed with a platinum loop, and, without recharging the loop, five or six small tubes of broth were inoculated. These tubes were incubated for different periods of time, and at different temperatures, and were then killed by the addition of formalin and the application of heat, as described in earlier reports. The killed cultures were then tested against type and group antisera.

Table I shows the results of one such series of tests, in which 258 colonies were examined in this way.

The figures need little comment. Those strains which, when incubated for 16 hours at 22° C. reacted with the type serum only, tended to react with both sera when incubated for a longer time or at a higher temperature. So marked was this tendency that after 16 hours at 37° C. over 97 per cent. of the type strains had become so altered as to give mixed agglutination. Practically no difference was observed between those type strains which had been

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held in stock for several months, and frequently subcultured, and those type strains which had recently been isolated from the faeces or tissues of infected mice.

As regards those strains which, after incubation for 16 hours at 22° C. reacted with the group antiserum alone, prolongation of the time, or elevation of the temperature of incubation, had much less effect. The great majority reacted throughout with a group but not with a type serum. There was, however, some tendency for mixed agglutination to occur, and this was more in evidence with the old stock strains than with those recently isolated.

Up to this point, the results could have been accounted for by assuming that type strains of *B. aertrycke*, when their growth in broth culture was

Table I.

*Showing agglutination results after growing B. aertrycke for different times at different temperatures and testing against a monospecific type serum and a group serum (Newport).*

Type strains	No. of strains tested	16 hrs. at 22° C.		16 hrs. at 37° C.		24 hrs. at 22° C.		24 hrs. at 37° C.		40 hrs. at 22° C.	
		Percentage of		Percentage of		Percentage of		Percentage of		Percentage of	
		T	M	T	M	T	M	T	M	T	M
Stock*	91	100	0	4.4	95.6	17.6	82.4	3.3	96.7	0	100
Recent	88	100	0	2.3	97.7	29.0†	71	0	100	4.5	95.5

Group strains	No. of strains tested	Percentage of		Percentage of		Percentage of		Percentage of		Percentage of	
		G		G		G		G		G	
		G	M	G	M	G	M	G	M	G	M
Stock*	34	100	0	73.5	26.5	82.4	17.6	79.4	20.6	85.3	14.7
Recent	45	100	0	93.3	6.7	100‡	0	100	0	100	0

T = Agglutination with *type* serum only.

G = " " " *group* "

M = " " " both sera.

\* The *stock* strains had been cultivated on artificial media for several months, or years. The *recent* strains had been isolated from faeces or tissues within a few days or weeks of the agglutination tests.

† 69 strains tested.

‡ 26 strains tested.

allowed to progress beyond a certain point, became altered in such a way as to react with group as well as with type agglutinin. Other series of tests, however, carried out in an exactly similar fashion, gave entirely different results. Cultures of type strains incubated at 37° C. for 48 hours or more, were agglutinated by the type serum alone, mixed agglutination being conspicuous by its absence.

It was obvious, from the grouping of the results, that some factor was involved common to all the tests of single series, and it was natural to examine more carefully the properties of the antisera employed. On the hypothesis put forward by Andrewes (1922), we had assumed that any antiserum, prepared against a member of the *B. paratyphosus* B group of bacteria, other than *B. aertrycke* (Mutton), would be a satisfactory source of group agglutinin acting on the latter organism. We had, by chance rather than by design, selected an

antiserum prepared against *B. aertrycke* (Newport), which agglutinated group strains of the Mutton bacillus to a high titre. We had, from time to time, substituted one batch of anti-Newport serum for another, when a given bottle was exhausted, each new batch being tested against several group strains of *B. aertrycke* (Mutton) to ensure the presence of an adequate amount of group agglutinin.

We now proceeded to test six such Newport sera, four paratyphosus B sera, four paratyphosus C sera, and three Gaertner sera against a group strain of *B. aertrycke* (Mutton), and against a type strain which had been grown at 37° C. for 40 hours. The results, set out in Table II, are somewhat surprising, but quite unequivocal. Clearly, the agglutination of the altered type strains, by

Table II.

*Showing titres obtained by testing various sera against group strains of B. aertrycke grown at 22° C. for 16 hours and against type strains grown at 37° C. for 40 hours.*

Serum*	Bacterial suspensions	
	Group	Type
	16 hrs. at 22° C.	40 hrs. at 37° C.
Newport 1	6,400	1,600
" 2	6,400	800
" 3	6,400	200
" 4	12,800	800
" 5	1,600	0
" 6	6,400	102,400
Paratyphosus B 1	3,200	0
" 2	3,200	0
" 3	1,600	0
" 4	1,600	0
Paratyphosus C 1	800	0
" 2	400	0
" 3	400	0
" 4	800	0
Gaertner 1	0	0
" 2	0	0
" 3	0	200

\* No serum gave any agglutination with type strains of *B. aertrycke* grown at 22° C. for 16 hrs.

the Newport antisera, is unconnected with the presence of group agglutinin in the serum or of group antigen in the bacillus. An entirely different antigen-antibody complex has come into play. Five of the six Newport sera have a titre against the group strain of *B. aertrycke* which is practically constant, but their titre against the altered type strain varies over an enormous range. Serum Newport 3 and serum Newport 6 each agglutinates the group culture to a titre of 1/6400, but serum 3 agglutinates the altered type culture to 1/200, while serum 6 agglutinates it to 1/102,400. Again, the paratyphosus B sera contain considerable amounts of group agglutinin, but no trace of agglutinin for the altered type strain. The same is true for the paratyphosus C sera, though here the titres for the group strain are relatively low. The three Gaertner sera are almost without action, but it is of interest to note that,

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while none of them contains group agglutinin, serum Gaertner 3 does agglutinate altered type strains to a low titre.

It is clear that type strains, under certain conditions of growth, to which in practice they are very commonly subjected, become altered in such a way that they react with an agglutinin to which they did not respond in their unaltered form.

It is of interest to determine whether or no group strains undergo a similar alteration. With the exception of the weakly acting Gaertner serum, those sera which reacted with altered type strains also contained group agglutinin in considerable amount, but an altered group strain could be distinguished from an unaltered group strain by a change in the titre at which it reacted. Using, for instance, a paratyphosus B serum, which agglutinates group strains of *B. aertrycke* to 1/6400, but does not react with altered type strains, and also

Table III.

*Showing titres obtained by testing different group strains of B. aertrycke against a paratyphosus B serum and a Newport serum, after growth for different times at different temperatures.*

Strain*	SERUM			
	Paratyphosus B		Newport 6	
	16 hrs. at 22° C.	48 hrs. at 37° C.	16 hrs. at 22° C.	48 hrs. at 37° C.
G 1	3,200	3,200	8,000	64,000
G 2	3,200	3,200	8,000	32,000
G 3	3,200	3,200	8,000	64,000
G 4	1,600	3,200	4,000	64,000
G 5	1,600	3,200	4,000	128,000
G 6	1,600	3,200	4,000	128,000
G 7	3,200	1,600	8,000	128,000
G 8	1,600	1,600	4,000	64,000
G 9	3,200	3,200	8,000	128,000
G 10	3,200	3,200	8,000	128,000
G 11	1,600	3,200	4,000	64,000
G 12	1,600	1,600	4,000	128,000

\* All strains were tested against a type serum with negative results.

serum Newport 6, which has a titre of 1/6400 for group strains and 1/102,400 for altered type strains, we can grow group strains for various times at various temperatures and then test them against these two sera. If we find that a group strain, when grown under those conditions which produce antigenic alterations of the type strain, reacts with the Newport 6 serum to a markedly higher titre than 1/6400, while its agglutinability by the paratyphoid B serum is unchanged, then it would seem a safe conclusion that both type and group strains undergo the same antigenic alteration in broth cultures. Table III shows that these conditions do in fact hold true. It would clearly be possible to obtain additional information on this point by adequate absorption tests.

We may then conclude that we have been dealing with two different phenomena. In addition to the type and group antigen, and the corresponding agglutinins, there is another antigen constituent, which for the moment we may refer to as the X antigen with its corresponding X agglutinin. Broth

cultures derived from a type or group colony of the same organism and grown under suitable conditions contain type or group antigen alone, except in so far as, in either case, a few bacilli of the alternative serological variety may be present. If the time of incubation be prolonged, or the temperature be raised, the X antigen begins to appear in either the type or the group culture, and is thereafter present in addition to the type or group antigen.

From the results of the present series of experiments it would appear that the distribution of type agglutinin, group agglutinin and X agglutinin is somewhat as follows:

(a) A serum prepared against *B. aertrycke* (Mutton), and absorbed with other organisms of the same general bacterial group, contains type agglutinin, but neither group agglutinin nor X agglutinin.

(b) A group serum, *i.e.* a serum reacting with young broth cultures of group strains of *B. aertrycke* (Mutton), appears to vary according to the organism against which it is prepared.

(c) Sera prepared against group strains of *B. paratyphosus* B contain group agglutinin, but no X agglutinin and *ex hypothesi* no type agglutinin for *B. aertrycke* (Mutton).

(d) Sera prepared against group strains of *B. paratyphosus* C also contain the group agglutinin alone.

(e) Sera prepared against group strains of *B. aertrycke* (Newport) contain group agglutinin, and may or may not contain X agglutinin. The amount of X agglutinin in different batches of anti-Newport serum varies over an enormous range and shows no constant relation to the amount of group agglutinin.

(f) Sera prepared against *B. Gaertner* contain no group agglutinin, but may contain some X agglutinin.

The question of the presence or absence of X agglutinin in type sera in general clearly needs further study. It would be natural to suppose that a serum prepared against a type strain, which had undergone antigenic alteration, would contain X agglutinin. This X agglutinin might well be removed by subsequent absorption with closely related bacteria which had themselves developed the X antigen. We have, however, made no attempt to pursue this aspect of the question<sup>1</sup>.

What relation this X agglutinin may bear to the para-agglutinins or neben-agglutinins of certain workers, we need not now enquire. Clearly it is fundamentally distinct from the type and group agglutinins. So far as our results have significance, in this respect, they are in complete accord with the views which Andrewes has put forward.

It was clear that the occurrence of mixed agglutination in the broth cultures, with which we had been dealing, had entirely misled us with regard

<sup>1</sup> The results of some recent observations, not connected with the present investigation, suggest that the phenomenon of mixed agglutination with cultures grown at 37° C. occurs with many organisms of the paratyphoid-enteritidis group of bacilli.

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to the nature of the viable bacteria which those cultures contained. It seemed desirable to determine, as far as possible, the actual constitution of cultures which agglutinated with type serum, with group serum or with both.

An analysis of this kind was attempted by taking broth cultures, which had been tested by agglutination after growth at 22° C. for 16 hours, and preparing plate cultures from them. Next day 20 to 50 colonies from each such plate were subcultured into tubes of broth, these were incubated for 16 hours at 22° C., killed and tested by agglutination. Table IV shows the results obtained with 30 type strains, 30 group strains and 25 mixed strains. The results are quite clear-cut. Under such conditions, a culture agglutinating with a type serum alone gives rise to over 99 per cent. of type colonies when plated, a culture agglutinating with a group serum alone gives rise to over 95 per cent. of group colonies; while a mixed culture gives rise to both type and group colonies in not widely differing proportions, and mixed colonies

Table IV.

*Showing results obtained by plating from cultures, which had been tested by agglutination after incubation at 22° C. for 16 hours, and determining the agglutination reaction of many colonies from each plate.*

Agglutination of original culture	Number of cultures tested	Number of colonies tested*	Percentage of colonies agglutinating as		
			Type	Group	Mixed
Type	30	1464	99.5	0.4	0.1
Group	30	1465	2.7	95.2	2.1
Mixed	25†	464	36	53.9	10.1

\* 50 colonies were subcultured from each of the type or group plates: 20 from each of the mixed plates. A few colonies failed to give growth in subculture.

† 21 of the 25 mixed strains gave both type and group colonies on the same plate.

are relatively frequent. It seems clear that those cultures, which react with both type and group sera after growth at 22° C. for 16 hours, do so because they contain both type and group bacilli. The difference between the figures obtained with the type and group strains, though small, is, we believe, significant. It will be noted that among the colonies derived from type cultures only 0.4 per cent. react as group, while among the colonies derived from group cultures 2.7 per cent. reacted as type. This difference was very consistent over the 60 strains examined. For instance, 25 of the 30 type strains yielded colonies which were all type, while 12 only of the 30 group strains yielded colonies which were all group. We shall return to this difference later.

If now we turn to those cultures which give mixed agglutination after growing at 37° C. for 16 hours or longer, but which are known to have reacted during an earlier phase of growth as pure type strains, we obtain very different results. Table V gives such results with regard to 13 cultures. It is clear that the proportion of type colonies derived from a culture which has changed from type to mixed, as judged by agglutination, during relatively prolonged growth, does not differ sensibly from the proportion of type colonies derived from a culture which gives pure type agglutination. It would seem that the

X antigen first appears in those bacilli which are dead or dying, and so are unable to multiply further; or alternatively that, when an altered type bacillus containing the X antigen undergoes further division, it reverts to the non-X containing form.

It is clear that we may very easily avoid the source of error introduced by this antigenic alteration, by adhering strictly to 16 hours as the time, and 22° C. as the temperature of incubation, and that under these circumstances any group antiserum may be employed. Or, alternatively, we may select a group serum which contains no X agglutinin, and under these circumstances the time and temperature of incubation of the broth cultures may be varied within relatively wide limits.

When we adopt such precautions we find that cultures showing mixed agglutination are relatively very infrequent. During the past four and a half months we have tested 12,407 strains of *B. aertrycke*, recently isolated from the faeces or tissues of mice. Of these 7519 or 60.6 per cent. reacted as type,

Table V.

*Showing results obtained by plating from cultures which gave mixed agglutination after incubation for 16 hours or more at 37° C., but which had reacted as type after 16 hours at 22° C., and testing many colonies from each plate by agglutination.*

Number of strains	Number of colonies tested	Percentage of colonies agglutinating as		
		Type	Group	Mixed
13	239	98.8	0.8	0.4

4672 or 37.7 per cent. as group, and 216 or 1.7 per cent. as mixed. We have shown reasons for believing that those strains which, under these conditions, give mixed agglutination contain bacilli of each serological variety.

The results here reported offer an adequate explanation of the difference between our previous findings and those recorded by Andrewes, who claimed that the great majority of strains he examined reacted sharply either as type or group when tested by agglutination. Had we chanced to select any other group serum than that actually employed, we should have obtained the same results. The figures recorded above are entirely in accord with Andrewes' hypothesis.

It would seem, also, that variation from type to group, or *vice versa*, in artificial culture, is not such a rapid, irregular and unpredictable phenomenon as we had supposed. Table VI shows the results obtained in a short series of tests designed to yield information on this point. Eight sets of cultures were examined, four of type and four of group strains. In series T 1 and G 1, broth cultures of type and group strains respectively were continuously incubated at 22° C. and broth subcultures were daily prepared from them, grown for 16 hours at 22° C. and tested by agglutination. In series T 2 and G 2 similar broth cultures were daily subcultured to fresh broth, and then killed and submitted to agglutination: thus, this series represents a chain, or succession



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of subcultures in broth. Series T 3 and G 3 show the results of continuous incubation on agar, daily subcultures being made into broth for agglutination. Series T 4 and G 4 show the result of daily subculture from agar to agar, with the inoculation of parallel broth cultures from each agar tube, for the agglutination tests.

The figures given in the table represent the number of strains, examined on the day in question, which gave agglutination with both antisera, denoting that bacilli, of the alternative serological variety to that of which the original culture was composed, had begun to put in an appearance. Thus, on the sixth day, one of the 10 type strains subjected to prolonged cultivation on agar, gave rise to group bacilli, as well as type bacilli, on subculture; while eight out of nine group strains, which had been subjected to daily subculture from agar to agar, gave rise to both type and group bacilli. We should not attach any great significance to the results of this experiment, since the total number of strains included is small, but a few points seem clearly established. Within

Table VI.

*Showing the number of cases in which group variants appeared in type cultures, or vice versa, under various conditions of growth.*

Series	No. of strains	DAY								
		1	2	3	4	5	6	7	8	9
T 1. Broth continuous	13	0	0	0	0	0	—	0	0	0
T 2. „ subcultures	13	0	0	0	0	0	—	0	1	0
T 3. Agar continuous	10	0	0	0	0	—	1	0	0	—
T 4. „ subcultures	10	0	0	0	0	—	0	0	0	—
G 1. Broth continuous	7	0	0	0	0	0	—	1	2	3
G 2. „ subcultures	7	0	0	0	0	0	—	0	1	—
G 3. Agar continuous	9	0	0	0	0	—	0	0	0	—
G 4. „ subcultures	9	0	0	0	6	—	8	9	9	—

three days there is no evidence of serological variation in any case. Considering broth cultures alone there is no evidence of any serological variation within five days. The results of this experiment are in entire agreement with the view, based on the general results of many thousand tests, that, by subculturing a given colony to broth, killing the broth culture after a suitable period of incubation and testing it against the appropriate sera, we may obtain accurate information as to the character of the bacilli composing the colony in question.

It is clear from the table that the frequency with which type strains give rise to group variants is less than the frequency with which group strains give rise to type variants. In this connection we may recall that analysis of cultures, reacting as type and group respectively, showed that the frequency of occurrence of group bacilli in type cultures was less than the frequency of occurrence of type bacilli in group cultures. The table seems also to afford evidence that repeated subculture on agar is the most effective method of promoting serological variation. It seems possible that this may result from the increased probability of selecting chance variants, when some portion of a surface growth

is picked at random for subculture and this process repeated at frequent intervals.

THE EXCRETION OF *B. AERTRYCKE* IN THE FAECES.

In our previous studies on the faecal excretion of *B. aertrycke*, we were led to correlate the presence of group antigen with persistent faecal excretion. Since our method of determining the presence of group antigen was quite untrustworthy, our conclusions on this point as published are valueless.

It seems probable that the great majority of those strains which gave mixed agglutination were in reality altered type strains, but little purpose would be served by attempting any detailed analysis of our earlier results in the light of after knowledge. We may, however, safely make one assumption. In certain experiments we fed type strains to mice, and because we repeatedly isolated from the faeces strains which gave mixed agglutination, we assumed that we had failed in our object, and included those experiments with those in which group or mixed strains had been fed to the mice. Clearly this was

Table VII.

*Showing corrected figures for experiments recorded  
in a previous report.*

	Group strains No correction required	Type strains	
		As previously recorded	Corrected
Number of mice examined	35	35	50
Number of mice excreting <i>B. aertrycke</i>	20	4	14
Percentage of mice excreting <i>B. aertrycke</i>	57.1	11.4	28
Number of specimens examined	438	458	635
Number of specimens positive	70	6	42
Percentage of specimens positive	16	1.3	6.8

unjustified. We must classify these earlier experiments on the basis of the serological results obtained with the cultures used for feeding, irrespective of the agglutination results with the strains isolated from the faeces or from the tissues. If we do this we obtain a very different picture. Taking the figures given in Table I of the report referred to (Topley and Ayrton, 1924 *b*), we should transfer experiments *S* and *P* to the type class. The results of this transference are shown in Table VII, where the figures are compared with those set out in Table II of the earlier report. The figures, as emended, still suggest that feeding on group strains is more often followed by persistent excretion in the faeces, than is feeding on type strains; but there is not the sharp and apparently discontinuous variation which appeared to exist on the earlier basis of classification.

Clearly, however, it was necessary to repeat these experiments, using a technique free from the source of error which we had detected.

The general technique adopted did not differ from that already described (Topley and Ayrton, 1924 *a* and *b*) and it will suffice to recall a few of the more important details.

The broth cultures of *B. aertrycke* were administered by allowing drops to fall from a calibrated dropping-pipette into the open mouth of the mouse. Each mouse was subsequently housed in a separate cage, and observed for 42 days, or until its death if this occurred earlier. Specimens of faeces were examined on the 2nd and 3rd days after feeding, three times during each of the succeeding two weeks, twice during each of the three following weeks, and on the 41st and 42nd days. The number of viable *B. aertrycke* present was estimated by the method already described, and recorded on a logarithmic scale. An Excretion Coefficient, for any given series of specimens, collected from any series of mice, was calculated by dividing the total score recorded by the number of specimens in the series, and multiplying the result by 100.

All mice which died were examined post-mortem. Cultures were taken from the heart and spleen and, where a growth of *B. aertrycke* was obtained, plate cultures were prepared and 20 colonies from each plate were tested by agglutination. All surviving mice were killed on, or shortly after, the 42nd day. A portion of the spleen of each mouse was transferred to a tube of broth, these tubes were incubated for 48 hours, at 37° C., and any tube which showed growth was plated and examined in the same way as were the spleen cultures from those mice which succumbed to infection.

Tables VIII to XIII give a summary of the results obtained. Each table summarises four to five experiments. In each experiment five mice were fed on a type or a group strain. A few of these mice died before any faecal examination was made, and they are not included in the tables.

Tables VIII and IX refer to ten experiments in five of which type strains, and in five group strains were administered to mice, *per os*, in a single dose of

Table VIII.

*Showing results of feeding one large dose of type strain of*  
*B. aertrycke.*

Number of mice fed	...	...	...	...	...	...	...	29
Number of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	...	...	16
Percentage of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	...	...	55.2
Number of specimens of faeces examined	...	...	...	...	...	...	...	284
Number of specimens positive	...	...	...	...	...	...	...	44
Percentage of specimens positive	...	...	...	...	...	...	...	15.5
Excretion coefficient	...	...	...	...	...	...	...	64.4
Percentage of colonies from faeces reacting as	{	Type	...	...	...	...	...	84.0
		Group	...	...	...	...	...	13.3
		Mixed	...	...	...	...	...	2.7
Number of mice which died	...	...	...	...	...	...	...	16
Number of mice positive on post-mortem examination	...	...	...	...	...	...	...	16
Specific mortality per cent.	...	...	...	...	...	...	...	55.2
Percentage of colonies from tissues of dead mice reacting as	{	Type	...	...	...	...	...	93.8
		Group	...	...	...	...	...	5.3
		Mixed	...	...	...	...	...	0.9
Number of survivors killed on 42nd day	...	...	...	...	...	...	...	13
Number of survivors with positive spleen cultures	...	...	...	...	...	...	...	7
Percentage of survivors with positive spleen cultures	...	...	...	...	...	...	...	53.8
Percentage of colonies from spleen cultures reacting as	{	Type	...	...	...	...	...	98.6
		Group	...	...	...	...	...	0
		Mixed	...	...	...	...	...	1.4

Table IX.

*Showing results of feeding one large dose of a group strain of  
B. aertrycke.*

Number of mice fed ... ..	24
Number of mice which excreted <i>B. aertrycke</i> ... ..	17
Percentage of mice which excreted <i>B. aertrycke</i> ... ..	70.8
Number of specimens of faeces examined ... ..	289
Number of specimens positive ... ..	50
Percentage of specimens positive ... ..	17.3
Excretion coefficient ... ..	69.5
Percentage of colonies from faeces reacting as { Type ... ..	21.6
Group ... ..	75.6
Mixed ... ..	2.8
Number of mice which died ... ..	7
Number of mice positive on post-mortem examination ... ..	7
Specific mortality per cent. ... ..	29.2
Percentage of colonies from tissues of dead mice reacting as { Type ... ..	1.4
Group ... ..	81.4
Mixed ... ..	17.2
Number of survivors killed on 42nd day ... ..	17
Number of survivors with positive spleen cultures ... ..	13
Percentage of survivors with positive spleen cultures ... ..	76.5
Percentage of colonies from spleen cultures reacting as { Type... ..	80.4
Group ... ..	17.3
Mixed ... ..	2.3

0.04 c.c. of an 18 hour broth culture, grown at 22° C. Tables X and XI summarise 10 experiments, in which a single dose of 1/20th of this amount was employed, and Tables XII and XIII summarise eight experiments in which the latter dose, 0.002 c.c. was repeated on four separate occasions.

In these experiments the group strains did not lead to a markedly greater degree of faecal excretion than did the type strains. Some difference is apparent, and it is always a difference in this direction, but it is far less

Table X.

*Showing results of feeding one small dose of type strain of  
B. aertrycke.*

Number of mice fed ... ..	25
Number of mice which excreted <i>B. aertrycke</i> ... ..	4
Percentage of mice which excreted <i>B. aertrycke</i> ... ..	16
Number of specimens of faeces examined ... ..	361
Number of specimens positive ... ..	9
Percentage of specimens positive ... ..	2.5
Excretion coefficient ... ..	10.5
Percentage of colonies from faeces reacting as { Type ... ..	100
Group ... ..	0
Mixed ... ..	0
Number of mice which died ... ..	5
Number of mice positive on post-mortem examination ... ..	5
Specific mortality per cent. ... ..	20
Percentage of colonies from tissues of dead mice reacting as { Type ... ..	95
Group ... ..	5
Mixed ... ..	0
Number of survivors killed on 42nd day ... ..	20
Number of survivors with positive spleen cultures ... ..	6
Percentage of survivors with positive spleen cultures ... ..	30
Percentage of colonies from spleen cultures reacting as { Type... ..	71.7
Group ... ..	25.8
Mixed ... ..	2.5

Table XI.

*Showing results of feeding one small dose of a group strain of  
B. aertrycke.*

Number of mice fed	...	...	...	...	...	...	24
Number of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	...	7
Percentage of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	...	29.2
Number of specimens of faeces examined	...	...	...	...	...	...	333
Number of specimens positive	...	...	...	...	...	...	14
Percentage of specimens positive	...	...	...	...	...	...	4.2
Excretion coefficient	...	...	...	...	...	...	17.1
Percentage of colonies from faeces reacting as	{	Type	...	...	...	...	14.3
		Group	...	...	...	...	85.7
		Mixed	...	...	...	...	0
Number of mice which died	...	...	...	...	...	...	5
Number of mice positive on post-mortem examination	...	...	...	...	...	...	5
Specific mortality per cent.	...	...	...	...	...	...	20.8
Percentage of colonies from tissues of dead mice reacting as	{	Type	...	...	...	...	31.4
		Group	...	...	...	...	64.4
		Mixed	...	...	...	...	4.2
Number of survivors killed on 42nd day	...	...	...	...	...	...	19
Number of survivors with positive spleen cultures	...	...	...	...	...	...	7
Percentage of survivors with positive spleen cultures	...	...	...	...	...	...	36.8
Percentage of colonies from spleen cultures reacting as	{	Type	...	...	...	...	47.6
		Group	...	...	...	...	50.2
		Mixed	...	...	...	...	2.2

clear-cut and consistent than appeared to be the case in our earlier experiments, even when allowance was made for faulty technique. The figures which afford data for comparison in Tables VIII to XIII are the percentage of mice excreting *B. aertrycke*, the percentage of specimens of faeces yielding positive results, and the excretion coefficient. When one large dose of culture was administered (Tables VIII and IX) the figures for these values are all higher for the group than for the type series, though the differences are slight and of

Table XII.

*Showing results of feeding with repeated (4) small doses of type  
strains of B. aertrycke.*

Number of mice fed	...	...	...	...	...	...	20
Number of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	...	11
Percentage of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	...	55
Number of specimens of faeces examined	...	...	...	...	...	...	308
Number of specimens positive	...	...	...	...	...	...	40
Percentage of specimens positive	...	...	...	...	...	...	13
Excretion coefficient	...	...	...	...	...	...	44.1
Percentage of colonies from faeces reacting as	{	Type	...	...	...	...	98.5
		Group	...	...	...	...	1
		Mixed	...	...	...	...	1.5
Number of mice which died	...	...	...	...	...	...	4
Number of mice positive on post-mortem examination	...	...	...	...	...	...	4
Specific mortality per cent.	...	...	...	...	...	...	20
Percentage of colonies from tissues of dead mice reacting as	{	Type	...	...	...	...	95.6
		Group	...	...	...	...	3.1
		Mixed	...	...	...	...	1.3
Number of survivors killed on 42nd day	...	...	...	...	...	...	16
Number of survivors with positive spleen cultures	...	...	...	...	...	...	7
Percentage of survivors with positive spleen cultures	...	...	...	...	...	...	43.75
Percentage of colonies from spleen cultures reacting as	{	Type	...	...	...	...	97.9
		Group	...	...	...	...	2.1
		Mixed	...	...	...	...	0

Table XIII.

*Showing results of feeding repeated (4) small doses of group strains of B. aertrycke.*

Number of mice fed	...	...	...	...	...	...	...	20
Number of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	...	...	11
Percentage of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	...	...	55
Number of specimens of faeces examined	...	...	...	...	...	...	...	298
Number of specimens positive	...	...	...	...	...	...	...	48
Percentage of specimens positive	...	...	...	...	...	...	...	16.1
Excretion coefficient	...	...	...	...	...	...	...	56
Percentage of colonies from faeces reacting as	{	Type	...	...	...	...	...	13
		Group	...	...	...	...	...	83.75
		Mixed	...	...	...	...	...	3.25
Number of mice which died	...	...	...	...	...	...	...	4
Number of mice positive on post-mortem examination	...	...	...	...	...	...	...	3
Specific mortality per cent.	...	...	...	...	...	...	...	15
Percentage of colonies from tissues of dead mice reacting as	{	Type	...	...	...	...	...	42
		Group	...	...	...	...	...	55.3
		Mixed	...	...	...	...	...	2.7
Number of survivors killed on 42nd day	...	...	...	...	...	...	...	16
Number of survivors with positive spleen cultures	...	...	...	...	...	...	...	9
Percentage of survivors with positive spleen cultures	...	...	...	...	...	...	...	56.25
Percentage of colonies from spleen cultures reacting as	{	Type...	...	...	...	...	...	37.8
		Group	...	...	...	...	...	61.5
		Mixed	...	...	...	...	...	0.7

doubtful significance. In Tables X and XI, giving the results which followed the administration of one small dose of *B. aertrycke*, the differences are rather more marked, though the total number of positive results, upon which comparisons depend, is smaller throughout. With repeated small doses (Tables XII and XIII) the difference in the percentage of mice excreting, as between those fed on type and group strains, disappears. The percentage of specimens positive and the excretion coefficient are, however, higher for the group than for the type series. This indicates that, with repeated infection, while the proportion of mice which excrete *B. aertrycke* on one occasion at least is the same among those fed on type as among those fed on group strains; yet, among the latter, those mice which excrete tend to do so more persistently. The differences are so small that we should attach little significance to them, were it not for their entire consistency. When all the experiments are considered together, it seems to us that they do afford some evidence in favour of an increased frequency of faecal excretion following the administration of group strains as compared with type strains of *B. aertrycke*.

In calculating the proportions of the two serological varieties recovered from the faeces or from the tissues, under different conditions, certain adjustments have necessarily been made. It has been our routine procedure to subculture five colonies of *B. aertryke* from each faecal culture, when that number were available. On many occasions we have examined larger numbers of colonies, but frequently less than five colonies of *B. aertryke* have appeared on the primary cultures from the faeces. Each specimen examined should clearly exert an equal influence on the calculated results. For this reason we have allocated a total value of 5 to the agglutination results obtained with

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any one specimen of faeces, this figure being divided in accordance with the results obtained. From the tissues of mice which have died from infection, we have examined 40 colonies, 20 being taken from the heart culture and 20 from the spleen culture. From those mice which survived the experimental period and were subsequently killed, only 20 colonies have been examined, since spleen cultures alone were obtained. In a small proportion of cases some of the colonies from the plate have failed to grow in subcultures. In con-

Table XIV.

*Showing results of inoculating mice with small doses of type strains of B. aertrycke, intraperitoneally and subcutaneously.*

Number of mice inoculated	...	...	...	...	...	19
Number of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	5
Percentage of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	26.3
Number of specimens of faeces examined	...	...	...	...	...	120
Number of specimens positive	...	...	...	...	...	6
Percentage of specimens positive	...	...	...	...	...	5
Excretion coefficient	...	...	...	...	...	15.8
Percentage of colonies from faeces reacting as	{	Type	...	...	...	83.3
		Group	...	...	...	0
		Mixed	...	...	...	16.7
Number of mice which died	...	...	...	...	...	7
Number of mice positive on post-mortem examination	...	...	...	...	...	7
Specific mortality per cent.	...	...	...	...	...	36.8
Percentage of colonies from tissues of dead mice reacting as	{	Type	...	...	...	99
		Group	...	...	...	0.7
		Mixed	...	...	...	0.3
Number of survivors killed on 42nd day	...	...	...	...	...	12
Number of survivors with positive spleen cultures	...	...	...	...	...	9
Percentage of survivors with positive spleen cultures	...	...	...	...	...	75
Percentage of colonies from spleen cultures reacting as	{	Type	...	...	...	83.9
		Group	...	...	...	13.9
		Mixed	...	...	...	2.2

Table XV.

*Showing results of inoculating mice with small doses of group strains of B. aertrycke, intraperitoneally or subcutaneously.*

Number of mice inoculated	...	...	...	...	...	17
Number of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	6
Percentage of mice which excreted <i>B. aertrycke</i>	...	...	...	...	...	35.3
Number of specimens of faeces examined	...	...	...	...	...	119
Number of specimens positive	...	...	...	...	...	19
Percentage of specimens positive	...	...	...	...	...	16
Excretion coefficient	...	...	...	...	...	60.5
Percentage of colonies from faeces reacting as	{	Type	...	...	...	10.1
		Group	...	...	...	87.2
		Mixed	...	...	...	2.7
Number of mice which died	...	...	...	...	...	5
Number of mice positive on post-mortem examination	...	...	...	...	...	5
Specific mortality per cent.	...	...	...	...	...	29.4
Percentage of colonies from tissues of dead mice reacting as	{	Type	...	...	...	42.5
		Group	...	...	...	37
		Mixed	...	...	...	20.5
Number of survivors killed on 42nd day	...	...	...	...	...	12
Number of survivors with positive spleen cultures	...	...	...	...	...	11
Percentage of survivors with positive spleen cultures	...	...	...	...	...	91.7
Percentage of colonies from spleen cultures reacting as	{	Type	...	...	...	49.5
		Group	...	...	...	48.6
		Mixed	...	...	...	1.9

formity with the procedure adopted with the faecal cultures, we have allocated a total figure of 20 for the agglutination results, obtained with the cultures from any one mouse, in calculating the proportion of the two serological varieties isolated from the tissues.

We append summaries (Tables XIV and XV) of two small series of experiments, in which the faecal excretion of *B. aertrycke* was studied in mice which had been injected subcutaneously or intraperitoneally with small doses of this organism. These results appear to us to yield considerable support to the view that group strains of *B. aertrycke* are in some way better endowed than are type strains for multiplication in the intestinal tract.

If now we compare the proportion of the two serological varieties isolated from the faeces and from the tissues of dying or surviving mice, we find ample confirmation of the differences noted in our earlier experiments. In Tables VIII, X and XII, the percentages of type strains isolated from the faeces were 84, 100 and 98.5 per cent., from the tissues of mice which died 93.8, 95 and 95.6 per cent., and from the tissues of those mice which survived beyond the 42nd day 98.6, 71.7 and 97.9 per cent. In general, then, when mice are fed on type strains, the strains recovered from their faeces or from their tissues after death belong to the same serological variety.

In Tables IX, XI and XIII, the percentage of type strains are, for those of faecal origin, 21.6, 14.3 and 13 per cent., for those from the tissues of dead mice 1.4, 31.4 and 42 per cent., for those from the tissues of mice surviving beyond the 42nd day, 80.4, 47.6 and 37.8 per cent. In general then, when mice are fed on group strains, a certain proportion of type strains are recovered from the faeces and from the tissues; but, while the group bacilli markedly outnumber the type bacilli in the faeces, the proportion of type bacilli in the tissues may equal or exceed the proportion of group bacilli, and the relative frequency of the type strains in the tissues is greater in the case of surviving mice than in the case of those which succumb to infection.

A glance at Tables XIV and XV will show that similar facts hold true.

In Tables XVI and XVII are collected figures showing the relation between the proportion of type and group strains isolated from the faeces or tissues of any mouse, and the interval between the last administration of *B. aertrycke* and the day on which the strain was isolated. While there is little variation with any of the type series, the group series show a tendency for the group strains to be replaced by type strains to an increasing extent with lapse of time.

It would seem that mice which receive relatively large doses of group bacilli, either by the mouth or parenterally, and hence succumb rapidly to a general tissue invasion, yield in most cases strains of the same serological type from their tissues. If, however, the infection produced be subacute or chronic, then the majority of the group bacilli are eventually replaced by the type variety.

This progressive replacement of one serological variety by another may be



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due to some selective action of the tissues, tending to the preservation of type strains and the elimination of group strains of *B. aertrycke*. It may be that the tendency for the proportion of type strains in the faeces of mice, which have been fed on group strains of *B. aertrycke*, to increase with the interval which elapses between the feeding and the collection of a specimen of faeces for examination, may be a reflection of the selective action of the tissues from the sphere of whose influence the bacilli are returning to the intestinal tract.

Table XVI.

*Showing the percentage of type, group and mixed strains isolated from the faeces of mice fed on type or group strains of B. aertrycke, according to the time which had elapsed since feeding.*

Day	Strains administered							
	Group				Type			
	No. of specimens	Type	Group	Mixed	No. of specimens	Type	Group	Mixed
1-5	31	6.8	92.6	0.6	14	89.7	0	10.3
6-10	26	13.5	79.6	6.9	15	92.8	7.2	0
11-15	13	7.7	92.3	0	12	76.7	20.0	3.3
16-21	13	21.5	75.4	3.1	11	100	0	0
22-42	19	41	55.3	3.7	27	96.3	3	0.7

Table XVII.

*Showing the percentage of type, group and mixed strains isolated from the tissues of mice fed on or inoculated with, type or group strains of B. aertrycke, according to the time which had elapsed between administration of the bacteria and the death of the mice.*

Day	Strains administered							
	Group				Type			
	No. of mice	T	G	M	No. of mice	T	G	M
1-5	23	8.9	84.8	6.3	16	97.2	1.2	1.6
6-10	14	21.1	67.1	11.8	15	98.3	1.0	0.7
11-15	8	21.25	67.5	11.25	13	95.4	3.1	1.5
16-21	19	38.7	50.8	10.5	19	89.5	9.7	0.8
22-42	44	56.8	37.0	6.2	40	92.5	6.5	1.0

We must, however, consider the possibility that such results as those recorded above are the expression of an innate tendency for group strains to give rise to type variants, more readily than type strains give rise to group variants. We have already recorded experiments which suggest that such a tendency is, in fact, observable in cultures of *B. aertrycke* grown on the ordinary media of the laboratory.

The balance of probability is, perhaps, in favour of some selective action on the part of the tissues. In many cases mice, fed on group strains, have yielded spleen cultures giving only type bacilli on plating. Thus we must explain in some way the apparent disappearance of the group bacilli as well

as the appearance of the type bacilli. Again, there is no evidence, when type strains are fed to mice, that there is a tendency for group strains to appear in the tissues, and gradually to replace the type strains. The rate of production of variants by the two serological varieties in artificial culture does not appear to differ so widely as would be suggested by the results obtained *in vivo*. To account for these, apart from a selective action of the tissues, we should have to assume that the frequency of occurrence of group variants in type strains of *B. aertrycke* was of an almost negligible order.

It must, however, be remembered that we have not in these experiments employed cultures derived from single bacterial cells. Until we have more exact knowledge of the rate and direction of this particular type of bacterial variation, under many different conditions, we lack an essential part of the data required for arriving at a conclusion on the point at issue.

THE MINIMAL LETHAL DOSE OF CULTURES OF THE TWO SEROLOGICAL  
VARIETIES, AND OF ROUGH AND SMOOTH VARIANTS.

In our previous experiments, the serological reaction of different smooth strains of *B. aertrycke* was found to be uncorrelated with the minimal lethal dose of these strains, as judged by intraperitoneal injections. This finding has been confirmed in the experiments summarised in Tables XVIII and XIX.

The greatly increased minimal lethal dose of rough as compared with smooth strains, referred to in our previous report (Topley and Ayrton, 1924 *b*), was not invalidated by the error in our serological technique, and it appeared unnecessary to repeat these experiments.

Table XVIII.

*Showing results of inoculating mice with an 18 hours' broth culture  
of B. aertrycke.*

(Type.)

Dose in c.c.	Route	No. of mice	No. of mice died	Day of death	No. of mice survived	Percentage of mice survived
0.25	I.P.	2	2	1, 1	0	0
0.025	"	2	2	1, 1	0	0
0.0025	"	4	3	1, 5, 10	1	25
0.00025	"	4	4	1, 5, 6, 9	0	0
0.000025	"	4	1	1	3	75
0.0000025	"	2	1	7	1	50
0.00000025	"	2	0	—	2	100
0.25	Sc.	2	2	2, 5	0	0
0.025	"	2	2	4, 5	0	0
0.0025	"	4	3	2, 9, 6	1	25
0.00025	"	4	3	7, 10, 14	1	25
0.000025	"	4	3	12, 13, 19	1	25
0.0000025	"	2	0	—	2	100
0.00000025	"	2	0	—	2	100

Total number of mice inoculated = 40.  
" " " " died = 26.  
Percentage mortality = 65.

I.P. = intraperitoneal.  
Sc. = subcutaneous.

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Table XIX.

*Showing results of inoculating mice with an 18 hours' broth culture of B. aertrycke.*

(Group.)

Dose in c.c.	Route	No. of mice	No. of mice died	Day of death	No. of mice survived	Percentage of mice survived
0.25	I.P.	2	2	1, 4	0	0
0.025	"	2	2	3, 4	0	0
0.0025	"	4	4	2, 3, 5, 5	0	0
0.00025	"	4	3	5, 5, 7	1	25
0.000025	"	4	1	2	3	75
0.0000025	"	2	2	5, 8	0	0
0.00000025	"	2	0	—	2	100
0.25	Sc.	2	2	4, 5	0	0
0.025	"	2	2	4, 7	0	0
0.0025	"	4	3	2, 4, 11	1	25
0.00025	"	4	2	16, 17	2	50
0.000025	"	4	2	7, 9	2	50
0.0000025	"	2	0	—	2	100
0.00000025	"	2	1	7	1	50

Total number of mice inoculated = 40.

" " died = 26.

Percentage mortality = 65.

In the report already referred to, it was noted that the specific mortality following administration *per os*, did not differ significantly as between smooth type and smooth group strains, while the specific mortality following the administration of rough variants was definitely lower than when smooth strains were employed.

It will be noted that the figures recorded in Tables VII and IX of the present report suggest that the oral administration of type strains is followed by a higher mortality than is the oral administration of group strains. The actual course of events in the individual experiments summarised in these tables suggested that the apparent excess of mortality was due to an excessive death-rate in two small series of mice, and was probably not significant. To test this point further, two larger series of mice were fed on the same amount of culture, and the subsequent course of events was observed for 42 days, without examination of the faeces. The results are recorded in Tables XX and XXI, and show a slightly greater specific mortality with the group strains.

We may then accept the conclusion that the lethal effect of the administration of cultures of *B. aertrycke* varies sharply with the roughness or smoothness of the strain employed, but is uncorrelated with the presence of type or group antigen.

It should, perhaps, again be emphasised that, as we have never isolated rough strains during the course of experimental epidemics of mouse-typhoid, it is impossible to judge what rôle, if any, rough strains may play in the natural spread of infection.

Table XX.

*Showing results obtained by feeding 50 mice on type strains of B. aertrycke, one large dose (0.02 c.c. of an 18 hours' broth culture) being administered.*

Number of mice fed	...	...	...	...	...	...	...	50
Number of mice which died	...	...	...	...	...	...	...	17
Percentage of mice which died	...	...	...	...	...	...	...	34
Number of mice positive on post-mortem examination	...	...	...	...	...	...	...	11
Specific mortality per cent.	...	...	...	...	...	...	...	22
Percentage of colonies from tissues of dead mice reacting as	{Type							90.9
	{Group							5.9
	{Mixed							3.2
Number of survivors killed on 42nd day	...	...	...	...	...	...	...	33
Number of survivors with positive spleen cultures	...	...	...	...	...	...	...	19
Percentage of survivors with positive spleen cultures	...	...	...	...	...	...	...	57.6
Percentage of colonies from spleen cultures reacting as	{Type							96.6
	{Group							3.4
	{Mixed							0

Table XXI.

*Showing results obtained by feeding 50 mice on group strains of B. aertrycke, one large dose (0.02 c.c. of an 18 hours' broth culture) being administered.*

Number of mice fed	...	...	...	...	...	...	...	50
Number of mice which died	...	...	...	...	...	...	...	15
Percentage of mice which died	...	...	...	...	...	...	...	30
Number of mice positive on post-mortem examination	...	...	...	...	...	...	...	14
Specific mortality per cent.	...	...	...	...	...	...	...	28
Percentage of colonies from tissues of dead mice reacting as	{Type							16.4
	{Group							66.3
	{Mixed							17.3
Number of survivors killed on 42nd day	...	...	...	...	...	...	...	35
Number of survivors with positive spleen cultures	...	...	...	...	...	...	...	21
Percentage of survivors with positive spleen cultures	...	...	...	...	...	...	...	60
Percentage of colonies from spleen cultures reacting as	{Type							42.9
	{Group							46.7
	{Mixed							10.4

## DOSAGE.

So far as the question of dosage is concerned, the general results of the present series of experiments do not differ from those already recorded. We have not, however, repeated the feeding experiments with progressively decreasing amounts of culture, so that we have not covered the same range of dosage as in our earlier studies.

From the figures given in Tables VIII to XI, it will be seen that the larger the dose of culture administered the higher is the proportion of mice which excrete *B. aertrycke* in their faeces, the greater the death-rate, and the greater the proportion of surviving mice which harbour *B. aertrycke* in their tissues. With repeated doses (Tables XII and XIII) together amounting to 1/20th of the single large dose, the excretion figures are far higher than with the single small dose, and fall very slightly short of the figures for the single large dose. The percentage mortality is, however, no higher than with a single small dose, and very definitely less than with a single large dose. The effect of repeated dosage, *per os*, with mouse-typhoid bacilli has recently been studied by Lange (1924), with results of the greatest interest. Our results, which

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afford no evidence of an increased mortality with repeated dosage, appear at first sight to be at variance with those which Lange records. It is, however, quite probable that the contradiction is no more than apparent. The size of the doses administered, and still more perhaps the number of doses and the intervals between them, are probably of decisive importance. In any case the issues raised by Lange's results are of such importance, that it seems wiser to postpone any further discussion of this question until further data are available.

### SUBACUTE, CHRONIC AND LATENT INFECTIONS.

We have commented, in several previous reports, on the frequency of subacute, chronic, and latent infections among experimentally infected mice, especially as evidenced by the isolation of *B. aertrycke* from the spleens of animals which have survived for more than 42 days after infection. The extensive series of mice studied in our previous investigation enabled us to give figures showing the frequency of this type of infection in relation to the dose of bacteria administered (Topley and Ayrton, 1924 b). The series of experiments here reported has added considerably to the data available for the study of this condition. The results are summarised in Table XXII, and it will be noted that they entirely confirm those already recorded.

Table XXII.

*Showing the proportion of mice, surviving beyond a definite period after inoculation or feeding with cultures of B. aertrycke, which yielded cultures of that organism from their spleens.*

Series	History of mice	No. of mice	No. with positive spleen cultures	Percentage with positive spleen cultures
1	Survived for 21 days after intraperitoneal or subcutaneous inoculation	28	24	85.7
2	Survived for 42 days after receiving one large dose of <i>B. aertrycke</i> per os	98	60	61.3
3	Survived for 42 days after receiving one small dose of <i>B. aertrycke</i> per os	39	13	33.3
4	Fed on 4 small doses of <i>B. aertrycke</i> and survived 42 days after date of first feeding	32	16	50

We must conclude that latent infection is a frequent result of the administration of sublethal doses of *B. aertrycke* to mice. The condition would appear to involve a well-established equilibrium between parasite and host, for the mice show no evidence of ill-health during life, and when killed they show, in most cases, no recognisable lesions at autopsy. There may be some slight splenic enlargement, but in the great majority of cases the size of the spleen lies within the limits which we must regard as normal for the mouse. The growth of *B. aertrycke*, when a portion of the spleen tissue is incubated in nutrient broth, is the only evidence that any form of infection exists.

THE VIRULENCE OF STRAINS OF *B. AERTRYCKE* ISOLATED FROM SURVIVORS.

It is of some interest to determine the minimal lethal dose of cultures of *B. aertrycke* derived from the spleen tissue of these surviving mice. It would afford some explanation of the tolerance of the mouse tissues for the bacteria which they harbour, if we could demonstrate that these had become so modified as to be less virulent than the strains originally administered. It has indeed frequently been suggested that some such change does occur in bacteria which have passed into the tissues, and there vegetated. Many workers have reported experiments bearing on this point. So far as our own results are concerned we have obtained no evidence pointing in this direction.

We have tested strains of *B. aertrycke* isolated from the spleen of mice, which had survived for 42 days after infection, had shown no sign of ill-health, and had presented no lesions indicative of infection at autopsy. In order that the opportunities for reversion to a virulent type in artificial culture should be reduced to a minimum, we employed for inoculation the primary broth cultures, containing the portions of spleen tissue, after 18 hours' incubation at 37° C. The fact that the cultures contained *B. aertrycke* was determined by the preliminary withdrawal of a small sample of the culture fluid, which was tested by agglutination. The purity of the cultures was confirmed by subsequent plating. The results are shown in Table XXIII.

Table XXIII.

*Showing results obtained by inoculating mice intraperitoneally with direct broth cultures from the spleens of mice which had survived for 42 days after feeding with B. aertrycke.*

Dose	Strain							
	T 5		G 5		T 6		G 6	
	Day		Day		Day		Day	
0.25	1	1*	1	1	1	1	1	1
0.025	1	4	2	1	2	2	2	2
0.0025	4	6	6	1	8	5	5	6
0.00025	3	4	4	12	4	12	4	8
0.000025	5	5	6	6	S	S	S	10

\* Two mice were inoculated with each dose. The figures given indicate the day after inoculation on which each mouse died. In every case the mouse presented typical lesions and *B. aertrycke* was isolated from the tissues.

S=survived for 21 days.

These strains are clearly possessed of the normal degree of virulence, as tested by intraperitoneal inoculation. The equilibrium arrived at in the splenic tissue does not appear to be associated with any loss of virulence on the part of the parasite, once that parasite has commenced to multiply freely in a new environment.

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### THE PRESENCE OF AGGLUTININS IN THE BLOOD OF SURVIVING MICE.

In only one series of animals was this point examined. Of 68 mice which had survived for 42 days after being fed on a single large dose of *B. aertrycke*, six showed the presence of agglutinins in the blood serum. The titres obtained varied between 1/40 and 1/1280. The sera from the remaining 62 mice showed no reaction in a dilution of 1/20. All six mice with agglutinins in the serum gave cultures of *B. aertrycke* from the spleen. Of the 62 mice showing no agglutinins, 34 gave positive, and 28 negative spleen cultures.

We have seen that an equilibrium may be established between parasite and host, in virtue of which the bacteria may continue to subsist in the splenic tissues. We have failed to find any evidence that this equilibrium is dependent on a decrease of bacterial virulence: we have equally failed to demonstrate the presence of agglutinins in the great majority of those mice in which this equilibrium had been established, and, to this extent, we have failed to find evidence of an active immunity of the humoral type.

It must, however, be recognised that the absence of agglutinins does not exclude the presence of other anti-bodies. Wolf (1908) failed to demonstrate agglutinins in the serum of mice immunised by living cultures of mouse-typhoid bacilli, administered *per os*, but was able to show that antibodies were present which gave rise to the Pfeiffer phenomenon when mixtures of mouse-serum and bacterial suspension were inoculated intraperitoneally into guinea-pigs.

### SUMMARY.

We may summarise our results, including those recorded in our two recent reports (Topley and Ayrton, 1924 *b* and *c*) under the following headings.

(1) *The antigenic structure of B. aertrycke (Mutton).*

(a) *B. aertrycke* (Mutton) undergoes natural dissociation into two sharply distinguished varieties, one containing that antigenic constituent which is characteristic of this bacterial species or variety, the other that antigenic constituent which *B. aertrycke* (Mutton) shares with the bacterial group to which it belongs. We have obtained no evidence that both the type and group antigen may be fully developed in one and the same bacillus. In these respects our results confirm entirely those recorded by Andrewes (1922).

(b) Both the type and the group varieties of this organism, when grown for more than 16 hours at 22° C., or for that length of time at 37° C., tend to undergo an antigenic alteration such that they become agglutinable with an entirely different type of antibody, to which they did not previously respond. The antigen developed as a result of this alteration would seem to be the same for the type and the group varieties. Since plating from such altered cultures gives colonies of the usual type or group variety, it would seem probable that the altered bacilli are dead, dying or senescent organisms.

(c) Although type or group cultures readily give rise to individuals of the alternative antigenic variety in ordinary laboratory media, yet this change is

neither so rapid nor so unpredictable in its occurrence that it is impossible to determine the nature of any given colony, or to obtain a culture of the serological variety required.

(d) There is some evidence that type variants arise in group cultures more readily than do group variants in type cultures.

(2) *The relations between the presence of type or group antigen and other biological attributes of B. aertrycke.*

(a) Roughness and smoothness vary independently of the presence of group or type antigen. Rough forms may react as group or type.

(b) There is no difference between the virulence of the type and group varieties of *B. aertrycke*. While the virulence of all smooth strains is high, the virulence of all rough strains is low.

(c) There is some evidence that group strains of *B. aertrycke* give rise more readily to faecal excretion than do type strains, but the difference is not great. Rough strains appear, in this as in other respects, to be ill fitted for a parasitic existence, and do not give rise to persistent faecal excretion.

(d) There is a well-marked tendency for group strains, which have gained access to the tissues, to give place to the type variety. The longer the sojourn in the tissues, the more complete is this replacement. Type strains, under similar conditions, do not tend to be replaced by group strains.

There is a similar, but less marked, tendency for group strains to be replaced by type strains in the intestinal canal.

(3) *The excretion of B. aertrycke in the faeces, after administration by the mouth, and its relation to other phenomena of infection.*

(a) The excretion of *B. aertrycke* in the faeces, after administration *per os*, may be continuous or intermittent, or may not occur in a sufficient degree to be detected by the technique employed.

(b) The faecal excretion, in those mice which succumb to infection, is no more regular in its course than in those mice which survive. It is a frequent occurrence for mice to die with typical lesions of enteric infection, without ever having excreted *B. aertrycke* in detectable amounts.

(4) *The condition of mice which have survived the oral administration of B. aertrycke.*

(a) A high proportion of mice, which have survived for 42 days after the oral administration of *B. aertrycke*, yield cultures of this organism from their spleens when examined post-mortem. In many series of experiments the proportion of survivors showing such positive spleen cultures has exceeded 50 per cent.

(b) The strains of *B. aertrycke* isolated from the spleens of such survivors appear to be possessed of the normal degree of virulence.

(c) The presence of agglutinins in the blood serum can be demonstrated in only a very small proportion of such surviving mice.



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### (5) *The relation of dosage to the phenomena studied.*

(a) With a single dose, there is a definite relation between the number of viable *B. aertrycke* administered and the frequency of the phenomena indicating infection. The larger the dose the higher is the mortality rate, the more frequent and persistent is the faecal excretion, and the higher is the percentage of the survivors which harbour *B. aertrycke* in their tissues.

As the dose decreases there is, at first, a rapid fall in the frequency of these various phenomena, but when the dose falls below a certain limit further decreases produce relatively little effect, over the range of doses studied.

(b) Repeated administration of small doses of *B. aertrycke* results in a high frequency of persistent faecal excretion.

In conclusion we should wish to express our thanks to Miss E. R. Lewis, for her assistance during a large part of the investigations recorded above.

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## THE SPREAD OF BACTERIAL INFECTION.

### FURTHER STUDIES ON AN EXPERIMENTAL EPIDEMIC OF MOUSE-TYPHOID<sup>1</sup>.

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(With 2 Charts.)

In previous reports (Topley and Ayrton, 1924 *a*, *b* and *c*) we have recorded the results obtained by feeding mice on cultures of *B. aertrycke*, and studying the subsequent excretion of that organism in the faeces. The present report deals with the application of the same technique to the study of an experimental epidemic of mouse-typhoid.

#### PROCEDURE EMPLOYED.

The method adopted was as follows. Broth cultures of *B. aertrycke* were administered, *per os*, to a number of mice on three successive days. Their faeces were examined daily, and on the fifth day five mice were selected from whose faeces *B. aertrycke* had been recovered. These mice are referred to as F 1, F 2, etc. To initiate the epidemic these five mice were placed in an experimental cage of the type already described (Topley, 1923) and to them were added 20 normal mice, referred to as A 1, A 2, etc. On the following day, and on each subsequent day throughout the course of the experiment, one normal mouse was added to the cage. These mice were entered in the records as E 1, E 2, etc. All such normal mice had, before entry to the cage, been examined for the presence of *B. aertrycke* in their faeces with negative results.

A specimen of faeces was collected daily from each mouse, except on Sundays, or on those occasions when one or more of the mice refused to yield a specimen. These were examined according to the technique already described (Topley and Ayrton, 1924 *a*). All mice which died were submitted to the routine post-mortem examination, including the preparation of cultures from the tissues, the subculture of 20 or more colonies from plates yielding non-lactose fermentors, and the testing of these broth cultures against type and group agglutinating sera.

Three days after the termination of the experiment all surviving mice were killed. A specimen of blood was collected from each mouse for agglu-

<sup>1</sup> A Report to the Medical Research Council.

tination tests, and the serum was tested, in a long series of dilutions commencing at 1/20 against type and group suspensions of *B. aertrycke*. Each mouse was submitted to autopsy, and a tube of nutrient broth was inoculated with a portion of spleen tissue. From those spleen cultures which showed growth within 48 hours, plates of McConkey's medium were inoculated, and these plates were further examined as described above.

#### METHOD OF RECORDING RESULTS.

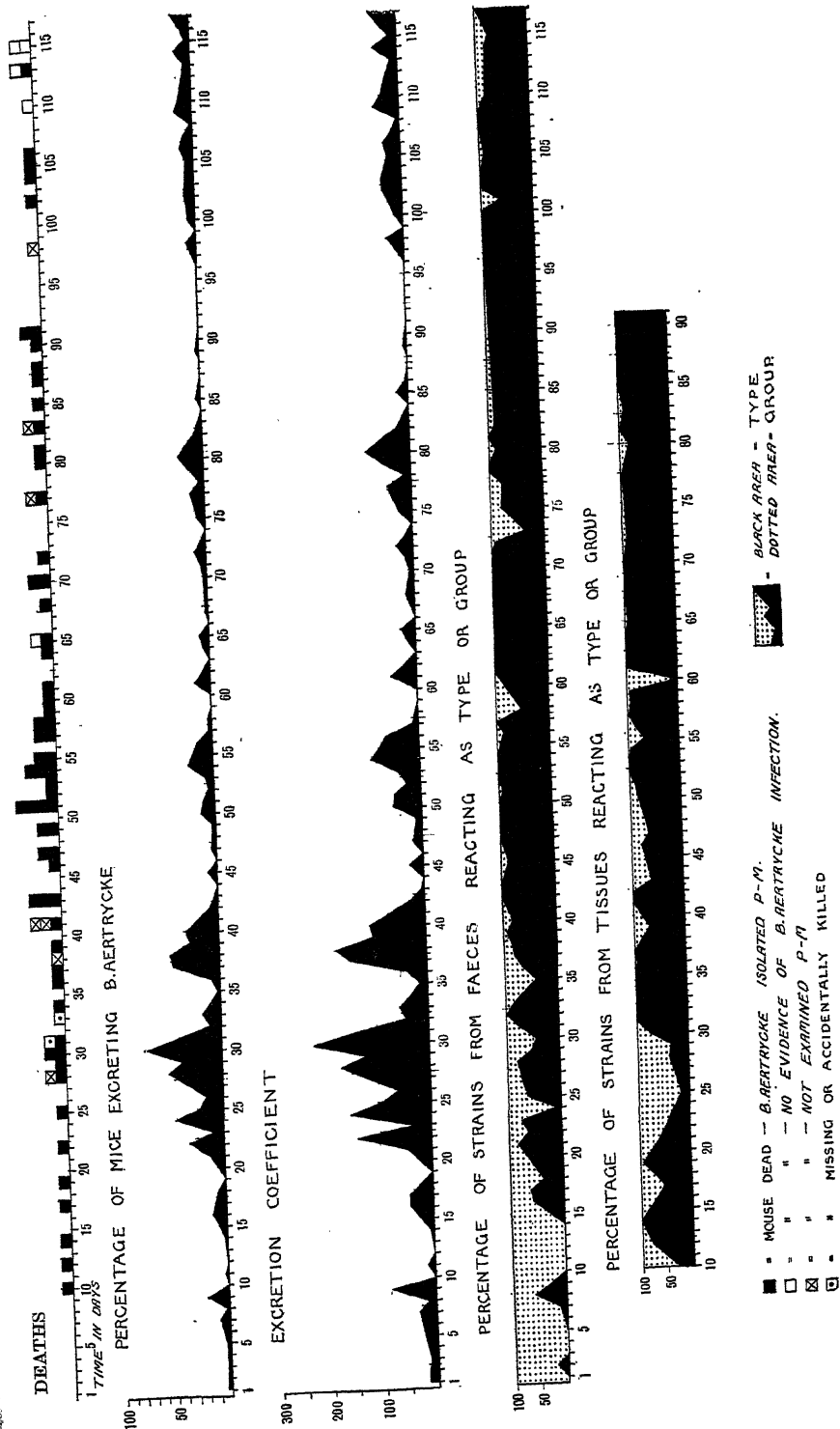
The significant results are recorded in Charts I and II and it would serve no useful purpose to include a detailed description in the text. It would, indeed, be difficult to prevent such a description becoming tedious and involved.

Chart I records the deaths, the daily excretion-rate, expressed as the percentage of the cage-population from whose faeces *B. aertrycke* was recovered on that day, and the excretion coefficient for that day's cage-population. This latter figure is the sum of the scores obtained by the excreting mice, allotted on the logarithmic scale already described (Topley and Ayrton, 1924 *a*), multiplied by 100 and divided by the number of specimens examined. It differs from the excretion-rate in that it allows for the relative copiousness of excretion of *B. aertrycke* by the individual mice. As will be seen, it appears to add no useful information to that obtained by a record of the excretion-rate.

In this chart are also included the results of the agglutination tests with the strains of *B. aertrycke* isolated from the faeces, or from the tissues after death. They are charted as percentages of type or group strains isolated on any given day. The percentages for the faecal and tissue strains are recorded separately in diagrammatic form.

In recording the results of these agglutination tests certain corrections have been made, in the light of our present knowledge of the errors introduced by factors which have recently been investigated (Topley and Ayrton, 1924 *c*). This investigation has shown that the very great majority of strains of *B. aertrycke*, recently isolated from the faeces or tissues of mice, can be sharply differentiated into Type or Group varieties by agglutination tests. There is a high probability that those strains which were found, in an earlier investigation, to agglutinate with both type and group sera were in fact altered type strains. During the first 39 days of the present experiment we were in ignorance of these facts. In this period 781 strains were isolated from the faeces and tested by agglutination. Of these, 330 reacted as type, 305 as group and 146 as mixed. During the remaining 78 days 591 faecal strains were examined, with adequate precautions. Of these, 533 reacted as type, 52 as group and 6 as mixed. It was clearly necessary to allow in some way for the errors introduced by faulty technique. There was, *a priori*, every reason for believing that all, or almost all, those strains which had reacted as mixed should be regarded as altered type strains. This probability was much strengthened by a consideration of our records. We may divide the first

Chart I.

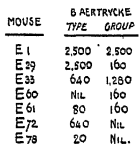


39 days of the experiment into three periods. During the first 14 days, 61 faecal strains were tested. Of these, 9 reacted as type, 47 as group and 5 as mixed. Thus, during a period when group strains were certainly being excreted in far larger numbers than type strains, mixed strains were relatively infrequent. Between the 14th and 25th days we examined 209 strains. Of these 60 reacted as type, 110 as group and 39 as mixed. Thus, during a period when the frequency of the type strains was increasing relatively to that of the group strains, the proportion of mixed strains was also increasing. Between the 25th and 39th days we examined 511 strains. Of these, 261 reacted as type, 148 as group and 102 as mixed. Thus, during the period in which the frequency of type strains exceeded the frequency of group strains, the frequency of mixed strains was at its maximum. Closely similar results were found to obtain in connection with the strains isolated from the tissues. It appears that the nearest approximation to truth will be obtained by regarding all the mixed strains as being, in reality, altered type strains; and this is what we have done. There would be no change in the general form of the diagrams, if we neglected all strains recorded as mixed, or introduced a separate shaded area to indicate those strains which were originally recorded under this head.

Two minor adjustments of another nature have also been made. It will be clear that the percentages charted on any date must be based on a very varying number of observations, according as many or few mice were excreting on the day in question. Where the number of colonies of *B. aertrycke* in cultures from any one specimen exceeded 5, that number of colonies were subcultured for agglutination tests. In many cases less than five colonies of *B. aertrycke* were obtained from a given specimen. The number of strains examined on a single day actually varied between 1 and 101. The significance attaching to the percentages recorded for the two serological types will clearly vary accordingly. On two occasions, once on the 11th day and once on the 89th day, a single strain was isolated during an interval in which the results were otherwise negative over several days. These two strains differed in serological reaction, from the strains which were isolated shortly before or shortly after them. To include these results as representing 100 per cent. of the serological variety in question, would introduce two large areas on the diagram suggesting a high frequency of these varieties over the corresponding periods. Since this is clearly unjustifiable, on the slender basis of a single agglutination test, these two results have been neglected in constructing the chart.

Chart II records the deaths, as in Chart I, and below this mortality base-line are recorded the results of the daily examination of faeces for each individual mouse. Each mouse is allotted a horizontal bar, extending from the day on which it entered the cage to the day on which it died, or on which it was killed at the termination of the experiment. These bars are shaded, or otherwise marked, to indicate the results of the daily examinations of the faeces. The signs employed to express these results, and also to record certain other observations, are fully explained on the chart.

## Chart II.



## DISCUSSION.

*Serological results.*

Dealing briefly with the serological results, Chart I shows clearly that, during the first phase of the spread of infection, group strains were far more frequently excreted than were type strains. In this connection it may be noted that the five infecting mice had been fed on strains of each variety. During the four days preceding the commencement of the main experiment, 29 strains of *B. aertrycke* had been isolated from the faeces of these mice, and of these 8 reacted as type and 21 as group. After about the 14th day, however, type strains rapidly increased in frequency, while the group strains diminished both relatively and absolutely. This phase corresponded to a period of increase in the percentage excretion-rate. The replacement of group by type strains continued thereafter, with few interruptions, throughout the whole of the experiment. There are some indications that periods, immediately preceding rises in the excretion-rate, are marked by an increased frequency of group strains; but we should not attach any great significance to this point, since the instances observed are neither striking nor numerous.

Of the replacement of group by type strains, during and subsequent to the epidemic period, there is no doubt. We know from previous experience that group strains, when fed to mice, give rise to faecal excretion somewhat more readily than do type strains; while type strains show a well-marked tendency to replace group strains in the tissues in sub-acute or chronic infections, and, to a less extent, to replace them in the faeces if excretion be long continued.

It is of some interest to find that a similar replacement of group by type strains occurs during an epidemic period; so that the one serological variety comes to predominate, among the population at risk, almost to the exclusion of the other. One can hardly avoid recalling the striking predominance of certain serological types in the epidemic spread of such diseases as pneumonia or cerebro-spinal fever in the human subject; but whether this apparent analogy has any real significance it is impossible at the moment to decide.

From the tissues of mice, which were killed at the termination of the experiment, 278 strains of *B. aertrycke* were isolated. Of these, 266 reacted as type, when tested by agglutination. Thus the results of the present experiment confirm our previous observations, with regard to the predominant rôle of the type variety in persistent tissue-infection.

The results of the agglutination tests, with the sera of those mice which survived beyond the 117th day, will be considered later. Reference to Chart II will show that, where agglutinins are produced, they may indicate a response to infection with either serological variety alone, or with both.

*Fluctuations in the excretion-rate.*

The daily determination of the percentage of excretors of *B. aertrycke*, among the population at risk, has enabled us to construct a continuous curve, showing the fluctuations in the excretion-rate during the entire course of the experiment. A consideration of this curve raises points of considerable interest. As will be seen in Chart I the excretion-rate begins to rise from about the 19th day of the experiment and attains a maximum on the 30th day, with minor fluctuations during its rise. It then falls sharply, commences to rise again about the 36th day, reaches a second peak on the 38th day, and then falls sharply again, maintaining a very low level between the 42nd and 49th days. Thereafter there are at least two minor but distinct fluctuations, with maxima on the 54th and 80th days, but the curve never again reaches the height obtained at the first or second peak. Towards the end of the epidemic there are indications of the rise of a fifth wave.

The first death is recorded on the 10th day, and thereafter sporadic deaths occur at frequent intervals, but the epidemic does not begin until about the 28th day, and does not reach its maximum until about the 51st day, after which it slowly declines, although occasional deaths continue to occur throughout the whole course of the experiment. The rise in the excretion-rate, therefore, precedes the rise in the mortality-curve, reaches its maximum during the early phase of the epidemic, and declines while the epidemic is still under way. The likeness to the course of events during the epidemic spread of cerebro-spinal fever is obvious and we may refer particularly to the observations recorded by Bruns and Höhn (1908).

The results obtained in this phase of the experimental epidemic afford an example of a generalisation which we believe to be of wide application: that, during the pre-epidemic phase of the spread of bacterial infection, there is a wide diffusion of the causative parasite among the population at risk, and that the proportion of hosts who are harbouring the parasite reaches a high level before the conditions requisite for an epidemic wave are attained.

The subsequent course of events raises another problem. The sudden decrease in the excretion-rate after the 30th, and after the 38th day, and the somewhat more gradual decreases after the 54th and 80th days are clearly shown in Chart I. How can we account for the fact that such a decrease may occur in a mouse population containing numerous excretors of *B. aertrycke*, when non-infected mice are present in the cage and fresh susceptibles are daily gaining admittance?

An examination of Chart II serves at least to narrow the problem. If the fate of individual mice be followed in this chart, it will be seen that we cannot explain the decrease in the excretion-rate by ascribing it to the removal, by death, of the excreting mice. It is clear that the rise of the excretion-rate to its first peak on the 30th day is due to the spread of the bacteria to hitherto uninfected mice. The chart shows, equally clearly, that the fall in the ex-



cretion-rate is due to the fact that the great majority of these mice cease to excrete at about the same time.

A careful study of the chart yields a clear demonstration that the fluctuations in the excretion-rate are due largely to intermittent excretion on the part of individual mice: A 1, A 9, A 14, E 12, E 13, E 26, E 29, E 33, E 36, E 52, E 53, and E 54 will serve as examples. Some factor seems to determine the approximately synchronous onset of excretion, and its synchronous cessation, in a high proportion of the population at risk; and individual mice respond to this factor by excreting, ceasing to excrete and excreting again in tune with their companions.

It is of some interest to consider in a little more detail the happenings during a single wave of excretion. If we take the period from the 20th to the 33rd day inclusive, covering the rise and fall of the first wave, we find that 52 mice were exposed to risk during some part of this interval. Of these, 42 mice excreted *B. aertrycke*. Of the 10 mice which did not excrete during this period, 4 were added on the last four days, so that their exposure to risk was of very short duration. There were 6 mice only which failed to excrete, though resident in the cage for the whole of the 14 days. Yet, on the 35th day, two days after the end of this period, only one mouse out of a cage-population of 43 was excreting *B. aertrycke* in detectable amount.

The data obtained in the course of earlier experiments, in which mice were fed on a single dose of *B. aertrycke*, throw some light on the question at issue. The observations recorded in the case of 219 mice are set out in Table I. The dose of culture fed to these mice varied widely in amount, but was in all cases relatively large, compared to the number of viable *B. aertrycke* likely to be ingested in a single dose during the natural spread of infection. The mice were observed for six weeks after feeding, or until death occurred at some earlier date, and 16 to 19 specimens of faeces were examined from each mouse which survived through the whole period.

When a mouse excreted *B. aertrycke* on more than one occasion, this excretion was often markedly intermittent; so that the table affords no data with regard to the actual duration of excretion. It does, however, show clearly that persistent excretion of *B. aertrycke*, after the ingestion of a single dose of that organism, is a rare event. It shows also that excretion is usually accompanied by tissue infection, and this is especially true with regard to those mice which excrete on more than one occasion.

It would seem to follow that a saprophytic multiplication of *B. aertrycke* rarely occurs in the intestinal tract of mice. Even when tissue infection is established it would appear to be unusual for *B. aertrycke* to secure a hold among the bacterial flora of the intestine.

It is not surprising then to find that a spread of *B. aertrycke* among the population at risk is followed by a phase of diminishing excretion. The rapidity of the fall in the excretion-rate, involving an almost simultaneous cessation of excretion on the part of all the infected mice, is not, however, easily ex-

plicable on the basis of our results with mice which have been infected by feeding. In such mice excretion is so irregular and intermittent that any curve, constructed by superposing the results obtained with 40 excreting mice, would show a slow and very irregular fall in the excretion-rate. It is difficult, in the light of our present knowledge, to account for the rapid and regular fall in the percentage of excreting mice, after each wave has reached its maximal point, on the basis of the reaction between the individual hosts and the parasites which they harbour.

An alternative hypothesis might be based on the assumed existence of some external factor, which affected all the mice in the cage simultaneously,

Table I.

*Showing the frequency distribution of 219 mice, classed according to the number of times B. aertrycke was isolated from their faeces, with the percentage of tissue infection observed in different classes.*

No. of isolations of <i>B. aertrycke</i>	No. of mice	No. of mice dying of <i>B. aertrycke</i> infection	No. of survivors with positive spleen cultures	Total No. of mice showing evidence of tissue infection	Percentage of mice showing evidence of tissue infection
0	127	22	24	46	36.2
1	33	14	8	22	66.7
2	18	9	9	18	100
3	11	5	2	7	63.6
4	11	7	3	10	90.9
5	6	4	2	6	94.7
6	2	0	1	1	
7	3	0	3	3	
8	1	0	1*	1	
9	1	1	0	1	
10	1	0	1	1	
11	1	0	1	1	
12	2	0	2*	2	
14	1	0	1*	1	94.7
17	1	0	1*	1	

\* Including one mouse kept alive for more than 42 days.

or in rapid succession; but we have, at the moment, no evidence that any such factor is involved. The solution of the problem must clearly await further investigation.

#### *Fluctuations in the Excretion Coefficient.*

In attempting to study the excretion of *B. aertrycke* in the faeces of mice, by a method which would yield quantitative results, having a relative significance in spite of a wide margin of error in the absolute values recorded, one of our objectives was to obtain data on the part played by dosage in the epidemic spread of enteric infection. It has already been suggested (Topley and Ayrton, 1924 *a* and *b*), that there is no simple conception of dosage, as a quantity which we can define and measure, under the conditions obtaining in an epidemic. The distribution of *B. aertrycke* in the total excreta of the mouse-population will determine not only the chance of some mouse ingesting a number of *B. aertrycke* falling within any given limits, but also the probable proportion of the population at risk which will ingest any *B. aertrycke* within

a given time-interval. If a few mice are excreting *B. aertrycke* in very large numbers, then it is likely that some mouse will ingest a large dose, while many mice will receive none. If a large proportion of the mice are excreting *B. aertrycke*, but none in great amount, then a high proportion of the total population will probably ingest some *B. aertrycke* within a given period, though none will receive a large dose. An extended experience of the actual course of events in individual mice shows conclusively that the variations in copiousness of excretion are in fact enormous. Is it possible to decide from the data before us whether mice which are excreting copiously play a preponderating rôle in the spread of infection?

The excretion coefficient differs from the excretion-rate in allowing for this factor of relative copiousness of excretion. If, in Chart I, we compare the curve showing the percentage carrier or excretion-rate with the curve showing the daily coefficient of excretion, it is clear that there is no possibility of differentiating between them, when attempting to correlate fluctuations in either with fluctuations in the curve of mortality. Their general form and the position in time of their maximal and minimal points are identical.

From the detailed records, not included in this report, we have noted the days on which specimens of faeces have been obtained giving a particularly high count of *B. aertrycke*, and have observed the fluctuations in the excretion-rate in the period immediately following. The results do not suggest that copious excretion on the part of individual mice is followed by a rise in the excretion-rate of the population in general. The highest count obtained in any one individual mouse, during the course of the experiment, was observed on the 22nd day, during the rise of the first wave of excretion, but after its commencement. The second highest count was observed on the 51st day, at a similar position on the third wave of excretion; but counts not insensibly inferior, coming indeed well within the wide limits of experimental error inherent in our technique, were observed on the 38th, 39th and 40th days, that is, during the early part of a fall in the wave of excretion, and preceding a prolonged interval marked by a low excretion-rate. Counts only slightly lower were observed on days 52, 53, 68, 81, 82, 85, 102, 103, and 105, a distribution which does not suggest any significant correlation with the other events observed.

There are many reasons for believing that repetition of relatively small doses may be a factor of crucial importance, and we may call attention in particular to some highly interesting observations recently reported by Lange (1924) and referred to in a recent report.

It appears to us that the whole question of dosage is too complex, and involves too many different factors, to permit of a useful discussion of its rôle in the epidemic spread of infection in the light of our present knowledge. A more extensive study of the individual factors concerned, under more strictly controlled conditions, may yield data which can be applied to the interpretation of such results as those here reported.

*The ratio of infected mice to the whole population at risk.*

Excluding the five mice infected by feeding, the population at risk during some part of the experimental period numbered 135. Of these, 122 yielded some evidence of infection.

There remained 13 mice, in which no evidence of infection was obtained by any of the methods of examination employed. Of these, 6 resided in the experimental cage for less than 14 days. One mouse, whose sojourn in the cage was 27 days, was eaten by his companions and could not be examined post-mortem. Thus, of 135 mice, which were exposed to risk for 14 days or more, only 6 failed to react in any way to the presence of the parasite, so far as could be determined by the methods of examination employed.

It appears that, under the conditions of this experiment, almost the whole of the population at risk played some part in the spread of infection.

*The fate of individual mice.*

In Chart II, it is possible to follow the fate of each individual mouse, from the time of its entry into the cage until its death, and to study the ways in which it reacted to the presence of the parasite.

The technique employed allows us to recognise four events which may be regarded as reactions to infection, using this term in a broad sense. A mouse may excrete *B. aertrycke* in its faeces. It may die, with or without the typical lesions of enteric infection, and yield cultures of *B. aertrycke* from its tissues. It may survive throughout the experimental period, but, when killed and submitted to post-mortem examination, yield a growth of *B. aertrycke* from its spleen. It may develop agglutinins to *B. aertrycke*.

A study of Chart II will show that the mice exposed to risk during the course of this experiment, afforded examples of most of the possible combinations of these criteria of infection.

*The mode of reaction of those mice which died during the course of the experiment.*

Excluding those mice which died from causes other than enteric infection, we may recognise certain well-defined modes of reaction, which have previously been noted in mice fed on cultures of *B. aertrycke*. A mouse may excrete this organism on several occasions, shortly before the fatal termination. Examples of this type of reaction are afforded by A 6, A 10, A 12, A 20, E 5, E 7, E 8, E 15, E 22, E 24, E 37, E 52 and E 90 (see Chart II).

Other mice may die of typical enteric infection without that organism ever having been isolated from their faeces, although specimens have been examined on many occasions. Examples are afforded by A 19, E 44, E 46, E 47, E 48, E 50, E 55, E 58, E 73, E 74, E 79, E 82, E 83, and E 94.

Among the former class are included certain mice, for example A 6 and A 10, which passed several weeks in the cage, during which they never

excreted *B. aertrycke* in detectable amounts, and then passed through a period of persistent excretion leading up to death. Mouse E 56, which died on the 119th day, affords a good example of this type of reaction. To the latter class might be added such mice as E 38, E 45 and E 57, which, after excretion on one or more occasions, passed through a long period during which they failed to excrete, before they succumbed to infection. .

Other mice excreted intermittently over long periods, so that they might have been regarded as chronic carriers, but eventually died with the typical lesions of enteric infection. Examples are afforded by A 4, A 5, A 13, E 3, and E 10.

A study of Chart II will show that there is some grouping of the first two classes, according to the time of their entry into the cage. The mice added between the 44th and 50th days, for example, include a notably high proportion of animals which succumbed to enteric infection without ever yielding cultures of *B. aertrycke* from their faeces. These mice were added subsequent to the two earlier and more marked waves of excretion, which affected the cage-population in general, and before the third wave had risen to any considerable height.

We cannot, it would seem, regard a definite period of faecal excretion as an essential, or even as a modal phenomenon in mouse-typhoid. There are no grounds for believing that the mice at risk become infected with *B. aertrycke*, pass through a definite incubation period, and then suffer from an attack of disease, conforming closely to a particular type, and ending in recovery or death. The fact that a mouse has excreted over a certain period, and then survived for many days or weeks without yielding positive cultures from its faeces, does not render it improbable that that mouse will later die with the typical lesions of the disease. A mouse which might be judged, on the evidence of prolonged but intermittent excretion, to have become a chronic carrier may come to a similar end.

Under the conditions of this experiment, that is under conditions which allow unhindered transference of the parasite to and fro from host to host, an acute attack of disease ending in death, or in recovery with acquired immunity, seems to play a minor part. The equilibrium between parasites and hosts, which must fluctuate as an epidemic wave progresses, appears to depend on many other factors.

*The mode of reaction of those mice which survived throughout  
the experimental period.*

The results to be considered under this head raise questions of considerable interest. On the 117th day, when the experiment terminated, 62 mice were living in the cage. Of these, seven died before the survivors were killed three days later, and these seven mice are not further considered.

The history of the remaining 55 survivors can be traced in Chart II, but the more important facts may be summarised as follows. From the spleen of

each of these mice a culture was made in nutrient broth and examined in the manner already described. From the spleens of 28 of the 55 survivors, cultures of *B. aertrycke* were obtained. In the remaining 27 cases the cultures were negative. The correlation between excretion of *B. aertrycke* during life and the isolation of *B. aertrycke* from the spleen after death is very low. Eighteen of the 55 survivors had never excreted *B. aertrycke* in detectable amount. Of these, 8 gave positive spleen cultures and 10 negative. Of the latter, one mouse had been in the cage for two days only, so that this result is without significance. Of the 37 mice which had excreted *B. aertrycke* on one or more occasions during life, 20 gave positive and 17 negative spleen cultures. Among the 20 mice, which excreted *B. aertrycke* and gave positive spleen cultures, the interval between the last occasion on which excretion occurred and the termination of the experiment varied between 0 and 63 days, with a mean interval of 16.25 days. For the 17 mice with negative spleen cultures this interval varied between 0 and 78 days, with a mean value of 21.5 days.

A specimen of blood was collected from each mouse immediately before it was killed, and was tested for the presence of agglutinins against *B. aertrycke*. Seven of the 55 survivors gave positive results, the titres varying from 1/20 to 1/2500. The sera of the remaining 48 mice gave no agglutination at a dilution of 1/20. Of the seven mice with agglutinins in the blood serum, three had positive spleen cultures and four gave negative results in this respect. Two of the seven mice had never excreted *B. aertrycke* during life and these had negative spleen cultures.

#### *The Factors concerned in Survival.*

We have summarised above the findings in 55 mice, which were living in the cage at the termination of the experiment, but we are mainly concerned with the condition of survivors. The duration of the exposure to risk, on that date, had varied between 2 and 117 days. Clearly the mouse which had been in the cage for two days cannot be regarded as a survivor. There are, indeed, no adequate grounds for selecting any particular period as significant in this sense; such a choice must be an arbitrary one. For the purpose of discussion we have taken an exposure to risk of 14 days or over as the point at which survival begins. The choice of any other adjacent limit would give essentially similar results.

Taking as attributes, the presence of *B. aertrycke* in the spleen tissue, the excretion of *B. aertrycke* during life, and the presence of agglutinins for this organism in the blood serum, we can construct 8 possible classes, and assign to each a proportion of the 46 surviving mice, which had lived in the cage for 14 days or more. The results are shown in Table II.

It will be seen that six mice had apparently failed to react in any way to the presence of the parasite, and reference to Chart II will show that these six mice were among the more recent entrants to the cage.

Of 40 mice which showed some evidence of infection, seven had agglutinins

in their serum. These results are in accord with those recorded in the preceding report, for mice fed on a single dose of *B. aertrycke* (Topley and Ayrton, 1924 c). We might be led to the conclusion that the formation of agglutinins is an unimportant mechanism in the survival of mice during the epidemic spread of enteric infection, but we do not think that such a view would be justified.

Webster (1922) was able to demonstrate the presence of agglutinins, active in a dilution of 1/200, or over, in 8 of 11 mice which had survived 30 feedings with heated cultures of mouse-typhoid bacilli, followed by a single intrastomachal injection of living culture. All 11 mice survived a subsequent intrapleural injection of many minimal lethal doses of living culture. It should be noted that the treatment by heat had apparently not sufficed to kill the culture used for the preliminary feedings, since 12 of 24 mice succumbed to infection with the bacillus employed.

Table II.

*Summarising the findings in 46 survivors which had lived in the experimental cage for 14 days or more.*

Class	Spleen culture positive	Agglutinins positive	Excretion positive	No. of mice
1	+	+	+	3
2	+	+	-	0
3	+	-	+	17
4	+	-	-	5
5	-	+	+	2
6	-	+	-	2
7	-	-	+	11
8	-	-	-	6

In a later experiment, in which he fed 18 mice on living cultures, 7 survived the preparatory period. These 7 mice were then given an intrastomachal injection of living culture. One of them died, without showing any lesions post-mortem. The other six survived, while 7 controls died with typical lesions. Of the 6 survivors, one contained agglutinins in the blood serum, active at a dilution of 1/200. All six mice survived a subsequent intraperitoneal injection with many times the minimal lethal dose of living culture.

In another experiment, Webster fed 50 mice for four weeks on a killed culture of mouse-typhoid bacilli. All agglutination tests were negative at the commencement of the experiment, and again one week after the feeding had ceased. The immunity of these mice was then tested, in 16 animals by intraperitoneal inoculation, and in 17 by intrastomachal injection. The results suggested some degree of immunity, but were far less clear-cut than in the preceding experiments.

Another series of 25 mice were fed daily on living cultures of mouse-typhoid bacilli. At the end of 30 days there were 18 survivors. Agglutination tests were negative at the commencement of the experiment, and again on the 13th day, but on the 36th day the sera of 7 of the 18 survivors gave agglutination at 1/200. All 18 mice were now inoculated intraperitoneally

with a living culture. Of the 18 mice, only four succumbed, and none of these had shown the presence of agglutinins.

Amoss (1922), in an experiment in which batches of susceptible mice were added at irregular intervals to an infected population, tested the blood of 56 survivors from one epidemic wave. During this wave a mortality of 69 per cent. had occurred among a population of 300 mice. He found that 37, or 66 per cent., showed the presence of agglutinins in their blood serum, giving some degree of flocculation at a dilution of 1/40. He records the further observation that 20 of these 56 mice agglutinated the bacilli completely at 1/40, while 36 agglutinated it partially or not at all in this dilution. Of the former one mouse died during the subsequent epidemic wave, while of the latter 8 succumbed. The protocols given do not show how these 8 mice were divided, as between those which gave partial agglutination at 1/40, and those which were completely negative.

Further data on this question are afforded by many experiments recorded by Webster in later reports (Webster, 1923, 1924) in which the agglutination tests were set up in progressive dilutions starting at 1/20. Webster himself summarises the results of his very thorough investigations by stating that: "In any series infected *per os* with a fixed dose, 20 to 30 per cent. show no sign of infection, no positive blood cultures, and no agglutinins; 5 or 10 per cent. present symptoms of disease, positive blood cultures, and then recover with or without homologous agglutinins; 70 or 80 per cent. develop positive blood cultures and succumb in a more or less constant ratio relative to time." Or, again: "The agglutination phenomenon is no criterion of immunity; a surviving mouse may or may not give a positive reaction."

A survey of the experimental results would seem to indicate that the more severe the immunising treatment to which any group of mice has been subjected, and the smaller the ratio of survivors to the whole population which has passed through a given experience, the higher will be the frequency of occurrence of agglutinins among the surviving mice. It is, however, clear from Webster's experiments that a well-marked resistance to infection may exist in the absence of agglutinins.

The small proportion of the surviving mice, in the experiment under discussion, which showed the presence of agglutinins in the blood, may be due to the fact that they were not survivors in the strict sense; that is, the epidemic had not been allowed to run its complete course. Had the addition of susceptibles been stopped, and the experiment been continued until deaths had ceased to occur, the proportion of mice showing the presence of agglutinins might have been very different.

Webster's observations yield powerful support for the view that, when an immunity reaction does occur, it is of that general type which is frequently associated with the formation of antibodies, and with their presence in the circulating blood. This conclusion is strongly supported by Neufeld (1924) in a recent critical review of the fundamental questions of active immunity,



which deals very fully with the available evidence in the case of mouse-typhoid infection.

Finally, we may consider the significance of our findings with regard to the presence of *B. aertrycke* in the spleen. This question has already been discussed in a preceding report (Topley and Ayrton, 1924 *c*) and it is of some interest to find that the results obtained, when mice are fed on cultures of *B. aertrycke*, are closely paralleled when they are exposed to the risk of natural infection. We can hardly doubt that the phenomenon of latent infection is of fundamental importance in the epidemic spread of disease; and its relation to the problem of superinfection, recently studied by Lange (1921) in the case of mouse-typhoid, has already been referred to.

We are not, however, in a position to discuss in any detail its real significance. It has been demonstrated that many mice, when infected by feeding or by exposure to risk during the course of an epidemic, contract a latent infection which may persist for many weeks at least, and probably for much longer. Our results give no indication as to whether such mice are more or less susceptible than their companions to the ingestion of further doses of living *B. aertrycke*.

The question has recently been considered by Webster (1924), but, in the absence of more detailed protocols, it is impossible to assess the real significance of his results. If we have not misread his account of the experiments in question, the demonstration that a mouse was suffering from chronic infection, at the time when it received a second dose of living culture, depended upon the isolation of two serological varieties of bacilli from the tissues after death. If this were so, it would clearly be impossible to detect the presence of latent infection when the bacilli employed for the preliminary treatment and for the subsequent infecting dose were serologically indistinguishable, and the results recorded suggest that this was frequently the case. Webster describes chronic infection in those cases where the preliminary treatment was carried out with his M.T. I bacillus or with *B. enteritidis*, strains which are serologically distinct from the M.T. II strain used in the subsequent test dose. The other strains employed for the preliminary treatment, with the exception of *B. paratyphosus* B, are, so far as we know, serologically indistinguishable from Webster's M.T. II strain, and with these no cases of chronic infection are recorded. On the evidence recorded it appears impossible to determine the proportion of mice which, at the time of the test inoculation, were harbouring living bacteria in their tissues. In the absence of this knowledge, we cannot compare the resistance of such mice with that of others, from whose tissues the bacillus was absent.

This problem is, indeed, beset with technical difficulties and until they are overcome we cannot arrive at any just conclusion.

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# A STUDY OF *C. DIPHTHERIAE* AND OTHER MEMBERS OF THE GENUS *CORYNEBACTERIUM* WITH SPECIAL REFERENCE TO FERMENTATIVE ACTIVITY<sup>1</sup>.

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## INTRODUCTION.

THE greater part of the work embodied in this paper and its main conclusions were placed at the disposal of the Bacteriological Committee of the Medical Research Council in connection with the recently published monograph on Diphtheria (*Diphtheria, its Bacteriology, Pathology and Immunology*, H.M. Stationery Office, 1923). It was considered by that Committee that the important subject of the fermentative activity of *C. diphtheriae* and of diphtheroids generally, demanded renewed enquiry and the writer was deputed to undertake the investigation under the supervision of Prof. J. C. G. Ledingham, a member of that Committee.

The monograph as published contains the gist of the data collated and conclusions reached but as, during the past twelve months the opportunity has been taken of adding materially to the number of strains submitted to

<sup>1</sup> For this work a grant was given by the Medical Research Council on behalf of its Bacteriological Committee at whose disposal much of the data here collated was placed.

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examination, it has been thought advisable to place on record this amplified report.

From Table I indicating source of material it will be seen that the additional strains (tabulated as Second Series) more than equal Series I discussed in the monograph.

Owing to the fact that the literature of the subject is fully dealt with in the Committee's monograph, it is unnecessary to refer to it here and the reader is therefore directed to the concluding chapter on "Diphtheroids" which contains ample information on this aspect of the subject.

### *Nature and source of material.*

The nature and source of the material examined is shown in Table I. All the strains in Series II and the majority of those in Series I were personally isolated from material sent to the Diagnosis Department of the Lister Institute. The remaining strains were obtained from the National Collection of Type Cultures. In both series when the source of the swab was not stated it was

Table I.

	Total No. of strains	Source			
		Throat	Nose	Ear	Other sources
Series I. <i>C. diphtheriae</i> Virulent	51	36	5	1	9
" II. " "	51	40	7	4	—
Series I. <i>C. diphtheriae</i> Non-virulent	17	17	—	—	—
" II. " "	14	9	4	1	—
Series II. <i>C. diphtheriae</i> Virulence not tested	35	32	3	—	—
Series I. Diphtheroids	79	36	10	5	28
" II. " "	71	47	15	9	—

assumed to have come from the throat. Although clinical particulars were lacking in some cases the virulent and non-virulent strains in both series were definitely known to include strains from suspected cases of diphtheria, from convalescents, healthy carriers and contacts. In Series I the virulent strains under the heading "other sources" included representatives of Bell's three serological groups; one old stock strain, one strain isolated from a sore on a barber's hand and four strains isolated by Minett from cases of ulcerative lymphangitis in the horse. Most of the strains of *C. diphtheriae* in Series II not tested for virulence were isolated to confirm doubtful morphological diagnoses in primary mixed cultures. When the morphology in pure culture, the type of growth on agar and the fermentation reactions were those of *C. diphtheriae* the nature of the organism was considered to be sufficiently indicated to justify or otherwise the original opinion and except in special circumstances the matter was not taken further. The diphtheroids isolated from human sources other than the ear, nose and throat include strains from the eye, cerebrospinal fluid, genito-urinary tract, chest-wall, abdomen and skin. Two strains were isolated from milk, one from a cat and another from an oyster.

*The securing of pure cultures.*

In a study of fermentation reactions purity of culture is obviously of prime importance. In dealing with corynebacteria the granular character of the emulsion made by many strains renders simple plating a less reliable means of obtaining presumably pure cultures than it is with organisms forming more homogeneous emulsions. Further, owing to the pleomorphism of some members of the genus some contaminations may readily escape detection in smears. In isolating strains for this work the primary mixed culture was plated in the case of Series I on inspissated horse serum. In Series II Douglas' tellurium trypsin agar was used for the great majority of the strains and found excellent. Colonies from the plates were picked on to agar. On this first slope it was usually possible to find an isolated colony for transfer to a second agar slope. If satisfactory this was used for inoculation of an inspissated serum slope and for preliminary fermentation tests. Agar was used at this stage and in the inoculation of all subsequent biochemical tests because an alteration in type of growth possibly due to contamination can be more readily observed on agar than on inspissated serum or other medium giving more profuse growth. This routine proved satisfactory in the majority of strains, though in some instances a series of five or six replates was made and in all cases a very careful look-out was kept throughout the investigation for any indication of possible contaminations.

*Preparation of medium used in fermentation tests.*

After a preliminary survey of some of the more recent literature on the fermentation reactions of corynebacteria and in particular of *C. diphtheriae*, glucose, maltose, galactose, saccharose, lactose, mannite and dextrin were selected for use in Series I. In Series II mannite was omitted. The medium used in both series was Hiss's serum water containing 1 per cent. of the carbohydrate with litmus as an indicator. After preliminary filtration of the serum the serum water mixture was heated for 30 minutes at 100° C. before the carbohydrate was added. When the carbohydrate and indicator had been added the tubes were heated for 10-15 minutes at 100° C. on three successive days. To test the sterility the whole batch was then incubated for two days at 37° C.

It was quite realised that the use of a carbohydrate containing medium sterilised by heat was open to criticism and the pros and cons were considered before the work was begun and again very carefully in the course of the investigation. The advantage which weighed most in its favour was the practical certainty of sterility, and though possible changes in the carbohydrate due to the heating were not lost sight of it was hoped that the error from this cause would be constant and that therefore the results obtained with different batches would be comparable. With the majority of carbohydrates used this was found to be so, for though slight variations in the

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degree of the reaction and the rate at which it was produced were observed in different batches these were not such as to lead to false conclusions. Unfortunately in the case of maltose and lactose the variations were more marked.

### *Anomalous fermentation reactions obtained with lactose.*

With lactose it was found in some batches of media that *C. diphtheriae* and some diphtheroids gave a definite acid reaction lasting 3-5 days or longer which as a rule gradually passed off and in 10-14 days or less had completely disappeared. With other batches the reaction was so incomplete and transient that it might have been passed over as of no significance. Maltose gave similar results with Group IV and some of the cane-sugar fermenting diphtheroids.

In search of a possible explanation of these variations the pH of several batches of media prepared in test tubes and in bulk was estimated electrolytically. With the exception of one batch which was tested only after standing for some months in a cold room at 0-5° C. all the batches had a reaction of 8.6 or 8.7. The initial reaction of the medium would therefore not account for the variations met with and the question of hydrolysis was then considered. As chemical analysis was not possible in the circumstances biological methods had to be relied on. Accordingly batches of heated and filtered media prepared from the same material at the same time were tested side by side from the point of view of lactose fermentation only. This experiment was very carefully controlled from many aspects. Some of the tubes contained litmus as an indicator but the majority were prepared without an indicator, cresol red being added to these at various dates after inoculation. Without going into the results in detail this experiment showed that *C. diphtheriae* and diphtheroids behaving like it on heated lactose containing media did not ferment lactose when the media were filtered. On the other hand one small group of diphtheroids which had previously given a definite and permanent acid reaction in all batches of heated media still gave an acid reaction when the medium was filtered, though this appeared more slowly than in the heated media. The latter must be considered to be a true lactose fermentation whereas the acidity produced by *C. diphtheriae* on heated lactose containing media can scarcely be so regarded. This view receives confirmation from an experiment in which both media were tested with *B. typhosus*. Litmus was used as an indicator in this experiment also which showed that *B. typhosus* behaved like *C. diphtheriae*, i.e. a definite but transient acid reaction was produced in heated media containing lactose but not in filtered.

As a result of these experiments the possibility of using filtered media on a large scale was reconsidered. Accordingly batches of filtered and heated media were prepared from the same material at the same time and all the carbohydrates used in this work were tested. Before use both batches were incubated at 37° C. for two days and were apparently sterile. Inoculations were then made with two representatives of each of the biochemical groups

about to be described as well as with strains of virulent and non-virulent *C. diphtheriae*. Readings were taken at the usual intervals.

In this experiment it was soon obvious that some of the diphtheroids grew much better on the heated than the filtered media though with *C. diphtheriae* and the more free growing diphtheroids the difference in growth in the two media was much less marked. As the slower rate of growth meant delay in the production of acid it was a distinct disadvantage and moreover it was disappointing to find that though one type of anomalous reaction had been avoided others had occurred. These affected maltose and dextrin and recurred when the experiment was repeated with fresh media. Though probably due to some fault in the samples of maltose and dextrin used they have not so far been satisfactorily accounted for as only the diphtheroid inoculated could be demonstrated in the affected tubes. As but for the previous results and present controls with heated media there would have been no reason to doubt the genuineness of the reactions produced with maltose and dextrin in filtered media the possibility of drawing false conclusions seemed more serious than with heated media. As the extent of possible error with the latter was now fairly well known the results about to be recorded are those obtained with heated media corrected especially in the case of lactose by the use of filtered material.

*Varying fermentability of different brands of dextrin.*

One other difficulty was encountered, that being the varying fermentability of different brands of dextrin. Owing to one stock of dextrin having been used throughout the investigation of Series I this variability was only observed later in the course of routine work when a fresh stock came into use. As the first sample had proved of very considerable value in differentiating *C. diphtheriae* from the diphtheroids, with Dr Robison's help some of the chemical as well as the biological characters of several brands of dextrin were examined. The results of this examination are given in detail in the Diphtheria Monograph and it need only be said here that no definite relationship could be established between the rotatory and reducing power of the different brands and the readiness with which they were attacked by *C. diphtheriae*. An interesting point brought out especially by the examination of the less sensitive samples was that some strains of *C. diphtheriae* were more active dextrin-fermenters than others. With such samples the majority of strains gave an incomplete acid reaction but at one end of the scale were a few which readily produced acid and clot whilst at the other were a small number in which the reaction was exceptionally feeble. Because of the uniform results obtained with the brand of dextrin used in the examination of Series I it has been included in the accompanying table of reactions but it cannot at present be considered a suitable substance for biological work.



*Recording the fermentation reactions.*

Before discussing the reactions obtained the method of recording these may be briefly noted. The carbohydrate containing-tubes were inoculated from 24-48 hour agar slopes and readings made daily for the first three days and subsequently on 5th, 7th, 10th and 14th days. On a few occasions 21 and 28 day readings were recorded. Contrary to custom when reading litmus tubes these were examined by transmitted light from a north window. Un-inoculated tubes were always incubated with the tests and used as controls. With *C. diphtheriae* and some of the diphtheroids definite though not always complete reactions were obtained in 24 hours. In the case of other diphtheroids, however, the reactions developed more slowly. With some strains this occurred with all the carbohydrates attacked but with others the delay was only observed in some of the test substances. In such cases it might be 3rd or even 5th day before the fermentation picture was complete. Even when the reaction had reached its maximum the degree of completeness was not necessarily the same in each carbohydrate. In some the reaction was limited to a colour change of the indicator whilst in others the change of colour was followed by the appearance of a coarse precipitate which might finally form a solid clot. Amongst themselves members of the same group might show slight variations in the strength of the reaction produced on individual carbohydrates but not infrequently even in detail the reactions of all members of the group were wonderfully uniform when the same batch of medium was used. The strains selected as types of the groups as well as many others have been examined at least five times with constant results. With very few exceptions all the strains in the first series have been completely tested at least twice and many have been under observation for two years or longer. Twenty-eight of the diphtheroids in Series II have been examined once only but the remainder have either been tested twice or oftener or two colonies from the same culture have been examined.

*Fermentation reactions of C. diphtheriae.*

The biochemical reactions of *C. diphtheriae* may be first considered. All the strains of virulent *C. diphtheriae* examined, 102 in all, gave the same reactions. Glucose, maltose and galactose were fermented. Glucose was most strongly attacked, clot being produced by all the strains. The tubes containing maltose and galactose were not invariably clotted and strains which produced clot in the one did not necessarily do so in the other. The reactions obtained with lactose and dextrin have already been alluded to at length. Cane-sugar and mannite were not fermented. Fermentation of cane-sugar by virulent strains of *C. diphtheriae* has been recorded by some writers but in this work when a virulent organism in all other respects indistinguishable from *C. diphtheriae* produced acid in cane-sugar it was always found that this reaction disappeared after replating even though in some instances no contamination could be demonstrated either microscopically or culturally.

Thirty-one strains morphologically and culturally indistinguishable from virulent *C. diphtheriae* gave exactly the same biochemical reactions but were not pathogenic for guinea-pigs. These must for the present be classed as non-virulent *C. diphtheriae*.

Table II.

Section	Group	No. of strains in group	No. of strains from nose and throat	No. of strains from ear	No. of strains from other sources	Glucose	Mal-tose	Galac-tose	Saccha-rose	Lac-tose	Mannite	Dex-trin
A	IV	9	7	0	2	+	±	-	-	-	-	-
	III	19	10	2	7	+	+	-	-	-	-	-
		28	17	2	9							
	V	13	9	2	2	+	+	+	-	-	-	-
	V <sub>a</sub>	2	2	0	0	+	±	+	-	-	-	-
		15	11	2	2							
B	II	16	13	1	2	+	±	-	+	-	-	-
	VII	8	3	0	5	+	+	-	+	-	-	-
	I	21	13	4	4	+	±	+	+	-	-	-
	VIII	11	10	1	0	+	+	+	+	-	-	-
	VI	7	7	0	0	+	+	+	+	+	-	-
	IX	1	0	0	1	+	+	+	+	+	-	+
	X	1	1	0	0	+	±	+	+	-	+	-
		65	47	6	12							
C	XI	42	33	4	5	-	-	-	-	-	-	-
		150	108	14	28							

*C. diphtheriae* (virulent and non-virulent)

*C. Pseudo-tuberculosis ovis*

" " *murium*

+ = acid production with or without clot.  
 ± in maltose column = transient acid production.  
 - = no acid production.

Table III.

Source and No. of the Diphtheroids in the Groups in

Group	Series I				Series II				Series I and II			
	Total	Nose and throat	Ear	Other sources	Total	Nose and throat	Ear	Other sources	Total	Nose and throat	Ear	Other sources
I	15	10	1	4	6	3	3	—	21	13	4	4
II	11	9	0	2	5	4	1	—	16	13	1	2
III	11	3	1	7	8	7	1	—	19	10	2	7
IV	6	4	0	2	3	3	0	—	9	7	0	2
V	4	1	1	2	9	8	1	—	13	9	2	2
V <sub>a</sub>	0	0	0	0	2	2	0	—	2	2	0	0
VI	4	4	0	0	3	3	0	—	7	7	0	0
VII	5	0	0	5	3	3	0	—	8	3	0	5
VIII	3	3	0	0	8	7	1	—	11	10	1	0
IX	1	0	0	1	0	0	0	—	1	0	0	1
X	1	1	0	0	—	—	—	—	1	1	0	0
XI	18	11	2	5	24	22	2	—	42	33	4	5
Totals	79	46	5	28	71	62	9	—	150	108	14	28

#### FERMENTATION REACTION OF THE DIPHTHEROIDS.

The fermentation reactions given by 79 diphtheroids in Series I divided them into 11 groups. With the exception of Group XI the numbers given to the groups correspond roughly to the frequency with which they occurred in

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Series I but the numbers in either series are of course too small to draw any conclusions as to the real incidence of the groups. Because of the variable and transient acidity already referred to produced in maltose by some strains, some of the groups are not sharply cut off from each other during the first days of the test. This applies to Groups I and VIII, II and VII, IV and III. In the first group of each pair the maltose reaction is variable in degree and always transient; in the second it is permanent and usually so strong that clot is formed. In Table II the biochemical reactions of the diphtheroid groups are given together with those of *C. diphtheriae*. In Table III the numbers in each group and the source are indicated.

As a discussion of the individual groups must involve consideration of their source, morphology and other characters it may be well here to examine these in relation not only to the diphtheroids but to *C. diphtheriae* as well.

### *Source in relation to fermentation reactions.*

Any further reference to the source of the various strains of *C. diphtheriae* examined is unnecessary. In the case of the diphtheroids it is found that strains from the nose and throat occur in all the biochemical groups except IX. All the larger groups and some of the smaller contain in addition strains from other sources. Urogenital strains are found in groups III, IV, V, VII, XI; strains from the eye in Groups I, II and XI and from the ear in I, III, V, VIII and XI. The source of the diphtheroid therefore gives no indication of the fermentation reactions to be expected.

Indeed on 18 occasions representatives of two biochemical groups were isolated from the same material and on three occasions the same swab yielded diphtheroids of three groups. Though in such cases one of the diphtheroids not infrequently (12 out of 21) belonged to the non-fermenting Group XI other combinations occurred. For example, Group VIII was found three times with Group I and once with Group III. Groups III and VII occurred together twice. In addition to these instances of diphtheroids of different groups occurring together two swabs yielded a pigmented and non-pigmented strain of Group XI. Though in one of these cases the non-pigmented strain might possibly be regarded as a variant of the pigmented one, in the other instance the morphology and type of growth on agar of the two strains were so different that they must be regarded as different organisms.

Some of the diphtheroids were also found in association with *C. diphtheriae*. Group XI was isolated six times, Group I twice and Group II once from the same swab as *C. diphtheriae*. This cannot, however, be taken as representing the true incidence with which *C. diphtheriae* and the diphtheroids occur together; for in routine work whether the isolation is being carried out for the purpose of a virulence test or to confirm a morphological diagnosis its chief aim is accomplished when *C. diphtheriae* has been found. On the other hand when only a diphtheroid is isolated the plate is fished much more thoroughly to exclude the possibility of *C. diphtheriae* being present as well.

*Growth on agar.*

The agar used was that in general use in the Institute and was made from a trypsin broth digest, pH, being adjusted to 7.8. On this medium the growth of *C. diphtheriae*, virulent and non-virulent, was usually a moderately fine one but it varied to some extent on different batches; the extremes of variation being a fine streptococcal-like growth and a very thick one almost like *B. coli*.

The latter has been found with only one batch of medium in the course of three years and is therefore of little practical importance.

The diphtheroids appeared on the whole to be less influenced than *C. diphtheriae* by the slight variations occurring in different batches of agar, the great majority being very constant in the character of their growth on this medium. The chief types of growth noted varied on the one hand from fine to moderately fine and on the other from rather thick to very thick. These two chief types were found in almost equal numbers among 79 diphtheroids of the first series whilst in the second thick growths predominated. The majority of the biochemical groups contain representatives of both fine and thick growths though in some groups one type of growth occurred more frequently than the other. Thick growths predominated in Groups I, V and VII and fine growths in II, whilst in III, IV and XI the two types occurred in almost equal numbers. The fine growths were almost colourless and the thick ones as a rule white or creamy.

(*Pigmentation is discussed on p. 250.*)

Stickiness of growth was noted in the majority of the strains in Group V and in a few strains in other groups particularly in Group III but it was often a somewhat elusive character and in some cases might easily have been missed if not specially looked for. These growths had often though not invariably a certain superficial appearance of dryness and at times were somewhat friable.

All the strains in Group VI and VIII had dry wrinkled growths which in the majority of cases were pigmented. One strain in Group IV and another in Group VII gave a very similar type of growth except that it was slightly thicker and more heaped up than wrinkled.

From time to time variant colonies have been observed on agar cultures but up to the present time has not permitted the carrying out of careful plating experiments with these. Sometimes but not always they appeared immediately after isolation. Such of these variants as it was possible to test at all gave the same fermentation reactions as the parent culture but as reliable cultures were not obtained the tests were inconclusive. Though the growth on agar of some diphtheroids is indistinguishable from that of *C. diphtheriae* the character of the growth on this medium as prepared in this laboratory has come to be considered in the course of this work a very useful point in distinguishing some of the diphtheroids from *C. diphtheriae*. A thick growth whether moist, dry, sticky or pigmented is very unlikely to be a culture of *C. diphtheriae* and with experience less marked variations from the

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typical diphtheriae like growth may excite suspicion as to the true nature of the organism.

### *Growth on serum.*

On inspissated horse serum *C. diphtheriae* gives a good growth, in the first 18–24 hours almost colourless but later becoming definitely white or creamy. The diphtheroids on this medium showed on the whole less individuality than on agar. Many of them gave growths very similar to *C. diphtheriae* but in some the growth was always thin and in others it was somewhat dry. But as with agar no type of growth was invariably associated with one group of fermentation reactions.

### *Pigmentation.*

This was studied only on agar and serum cultures and has been noted in 33 strains in all. In many cases it was shown equally well on both agar and serum but in some strains it was more marked and appeared more readily on agar. In some instances though very definite pigmentation was seen immediately after isolation, in subsequent subcultures pigment production was poor or only appeared at irregular intervals. With the exception of two very deep orange strains in Group XI the pigment shown has been a bright yellow often tending to an orange tint but in a few strains of a lemon shade. The incidence of the pigmented strains in the groups is shown in Table IV.

In the Diphtheria Monograph it has been pointed out that all the strains in Groups VI and VIII are pigmented. Among the additional strains placed in these groups in Series II one strain in group VI and three in Group VIII show very little pigment their colour as a rule not being more than a deep cream although their morphology and the general character of the growth apart from pigmentation is very like that of the other strains in the groups.

In the most markedly pigmented strain in Group VII the pigment is best shown on agar. The overnight growth on this medium is very fine and colourless but in three or four days becomes much thicker and shows a bright yellow pigment.

Pigmentation was not observed in any of the strains of *C. diphtheriae* examined.

Table IV.

Group	Source			Degree of pigmentation	
	No. of strains	Nose and throat	Other sources	Good, practically constant	Poor, inconstant or both
II	2	2	0	0	2
IV	3	2	1	2	1
V	1	0	1	0	1
Va	1	1	0	1	0
VI	7	7	0	6	1
VII	4	3	1	1	3
VIII	11	11	0	7	4
IX	1	0	1	1	0
XI	3	2	1	3	0

*Growth in broth.*

The broth used in this work was prepared in the same manner as that used as the basis for the agar medium. A few strains of *C. diphtheriae* grew badly on this medium but the majority gave good growths. In 48 hours the deposit was well marked, granular and distributed all over the bottom of the tube. The supernatant was granular or almost clear and a film was usually present. Most of the diphtheroids also grew well in this broth but in some cases the growth was poor. In a number of strains the growth resembled that of *C. diphtheriae* but in others, *e.g.* in Groups VI and VIII, though the general features of the growth were the same it was of a much coarser type. Still others gave a growth of a different character. The deposit though well marked was compact and confined to the centre of the bottom of the tube. The supernatant showed a general turbidity and a film might or might not be present. The character of the growth in broth therefore does not invariably distinguish the diphtheroids from *C. diphtheriae* and shows no constant relationship to their own fermentation reactions.

*Morphology.*

This was studied chiefly in smears made from 18–24 hour cultures on inspissated horse serum and stained with Löffler's methylene blue. Growths from agar were also examined and Gram's and Neisser's stains were used in some cases. The latter proved disappointing as a differential stain as many of the diphtheroids showed well-marked polar granules with it.

It is well known that the morphology of many corynebacteria varies with the age of the growth and the media on which they are grown, but variations in length, breadth and staining have also, not infrequently, been observed under apparently the same conditions. These variations occurred most commonly and were usually most marked immediately or soon after isolation. Occasionally the change in morphology has been so great as to throw doubt on the identity of the culture had not the biochemical and other characters remained unaltered. In these circumstances only a very general outline of the morphological types usually found will be given. In the first place these may be conveniently divided into very short forms and forms of medium length. Except in two recent batches of serum long or very long forms were not found as the predominant type in 18-hour serum cultures though they occurred on agar particularly with *C. diphtheriae*. Some of the very short strains were almost diplococcal and occasionally only the presence of a few longer or barred forms or the morphology on another medium made it possible to determine the real character of the culture. Members of these three primary morphological types may differ from each other in breadth or in the staining capacity of the protoplasm. The protoplasm may stain uniformly and deeply or it may stain lightly as a whole but show polar granules or deeply stained patches distributed regularly or irregularly throughout its length giving a barred or striated appearance. The outline of the organism may be clear cut

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and definite or hazy and ill-defined. Haziness of outline may be shown by the whole organism or more commonly, especially among the diphtheroids, it is seen only at one or both ends. One or both ends may be pointed, rounded off or more or less swollen. Swelling of the ends is much more frequently seen in *C. diphtheriae* than in the diphtheroids. A large number of types all passing gradually into each other are formed by varying combinations of the features just described. Though not infrequently particularly among the diphtheroids one morphological type predominates in a given strain it is rare even among those members of the genus to find a culture composed strictly of one type.

Contrary to the experience of some workers, in this investigation it has been found that as a rule the difference between the morphology on agar and on serum of members of the genus has been that on the former the organism is longer or broader on both. On the other hand, marked variations in morphology were on the whole commoner on agar than on serum.

Doubtless the chief interest and importance of the morphology of the diphtheroids lies in their resemblance or otherwise to *C. diphtheriae*. Though certain morphological characters such as polar granules, marked irregularity of staining and clubbing are commonly associated with *C. diphtheriae* representatives of practically every morphological type may be found in cultures of this organism. Commonly several types are present in the same culture and an atypical type may even predominate. On the other hand, in a culture of a diphtheroid composed chiefly of a type not at all characteristic of *C. diphtheriae* a few individuals are often found which by themselves are indistinguishable from *C. diphtheriae*. Thus the opinion has been formed as a result of this work that the distinction between *C. diphtheriae* and other members of the genus depends at least as much on the general impression made by the smear as on the presence of individual types; the marked pleomorphism and the great irregularity of the arrangement of individuals in relation to each other in *C. diphtheriae* being often in marked contrast to the comparative uniformity of individuals and the impression of orderliness given by the smear of a diphtheroid as a whole. Whilst in pure culture and in the majority of cultures made direct from swabs sent to the laboratory for diphtheriae diagnosis *C. diphtheriae* can usually be distinguished from most diphtheroids with comparative certainty; in some cases particularly in primary mixed cultures it may be very difficult if not impossible to give a definite opinion as to the nature of the organism on morphological grounds only. It should not be forgotten that some of the most difficult problems in the morphological diagnosis of *C. diphtheriae* in primary cultures may be due to organisms which are not members of the genus *corynebacterium*.

In the course of this work three or four diphtheroids have been encountered which have given more or less constantly a morphological picture quite indistinguishable from that of *C. diphtheriae*. The first of these was isolated from a cat suspected of causing diphtheria in a family. The second is the mouse organism *C. pseudotuberculosis murium*. Both of these ferment cane-sugar

and are not pathogenic to guinea-pigs. The morphology is therefore the chief point they have in common with *C. diphtheriae*. The third organism, *C. pseudotuberculosis ovis*, has not been so constant in its morphological resemblance to *C. diphtheriae* as the other two but on some occasions the resemblance has been very complete. It ferments glucose and maltose and some strains give a slight reaction with galactose. Cane-sugar is not fermented. In its biochemical reactions therefore it is more closely allied to *C. diphtheriae* than the other two organisms. In addition it is pathogenic to guinea-pigs but the control animal is not protected by an amount of diphtheria antitoxin which would be sufficient to protect against a similar dose of *C. diphtheriae*. The growth on agar is at first thin and dryish, later becoming definitely dry and wrinkled, a type of growth which has not been observed in any strain of virulent *C. diphtheriae*. There are grounds therefore for placing this organism also among the diphtheroids.

The fourth organism morphologically like *C. diphtheriae* will be considered when discussing Group XI.

#### *Virulence of C. diphtheriae and the diphtheroids.*

In Series I and in the majority of the strains in Series II the virulence of both *C. diphtheriae* and the diphtheroids was tested by the subcutaneous inoculation of a well-grown 48 hours broth culture. Sixteen strains of *C. diphtheriae* and six diphtheroids in Series II were tested by the intracutaneous method. In dealing with the few strains of *C. diphtheriae* which did not grow well on the ordinary broth used a good growth could always be obtained by the addition of ascitic fluid to the medium. In testing *C. diphtheriae* and the four strains of *C. pseudotuberculosis ovis* a dose of 2 c.c. was given to the test animal and 2.5 c.c. plus 200 units of antitoxin to the control. In the case of the diphtheroids the dose was 3 c.c. or 3.5 c.c. and no antitoxin controls were used. The weight of the guinea-pigs at the time of inoculation varied from 240–300 gms. Tested in this way virulent strains of *C. diphtheriae* caused death with characteristic post-mortem changes, in 24–72 hours. Deaths occurring later than this were either found to be due to some cause other than the inoculations, *e.g.* a Gaertner infection or pseudotuberculosis; or they fell into line with the others when a better grown culture was used.

The four strains of *C. pseudotuberculosis ovis* tested all caused death of both the test and the control animals in 24–48 hours. In two cases the protected animal lived a few hours longer than the unprotected but in the remaining two both animals died at practically the same time. Post-mortem all except one unprotected animal showed some degree of injection and oedema at the site of inoculation. The injection was always slight but in one unprotected and two protected animals the oedema was very marked. A varying degree of congestion was noted in various organs but macroscopic suprarenal changes when present at all were very slight compared with those usually found with *C. diphtheriae*. With two strains slight macroscopic changes were found in



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the suprarenals of the test animal whilst those of the control showed none, but with the other two strains the position was reversed.

The pathogenicity of 65 of the diphtheroids in Series I was tested. The strains examined included all the members of Groups III, IV and V, 35 cane-sugar fermenters and 10 of the non-fermenting organisms in Group XI. In the second series all the strains in Group V and *Va*, two strains in Group III and one strain in each of the Groups II and XI were examined for virulence. The animals were kept under observation for a month or longer. Nine of the animals died 10 days or more after the inoculation. In some of these no obvious cause of death was found on post-mortem examination whilst others showed evidence of a "Gaertner" infection or pseudo-tuberculosis. The experiment was repeated in all nine cases and all the animals survived. Though it can be said that the inoculation of guinea-pigs with large doses of diphtheroids causes no mortality some of the animals did not gain weight at the normal rate; about one-third gained less than 30 gms. in four weeks. Too much significance, however, cannot be attached to this observation as unfortunately no uninoculated controls were kept under the same conditions. The inoculations in Series I were made towards the end of August and in the first half of September there were rather severe morning frosts. It was thought that these might account in part at least not only for the failure to gain weight but also for some of the late deaths referred to above. The experiments at any rate show that the pathogenicity of the diphtheroids for the guinea-pig, if any, is on quite a different plane to that of *C. diphtheriae*.

### DISCUSSION OF SOME OF THE GROUPS.

Before concluding some points of interest in relation to one or two of the diphtheroid groups may be referred to. Though in discussing source, morphology and cultural characters it has been pointed out that no constant relationship was found between these and the biochemical reactions most of the groups contain strains which are indistinguishable from each other and may be regarded as examples of the same organism. This is a marked feature in Groups V, VI and VIII.

#### *Group V.*

The failure of dextrin as a reliable differential substance makes the position of the strains in this group of special interest, as apart from the absence of dextrin fermentation their biochemical reactions are those of *C. diphtheriae*. In Series I only one strain in this group was isolated from the nose and throat whereas out of a total of 13 in both series, nine are from nose and throat, two from the ear and one each from a whitlow and a genital swab. Eleven of the 13, all the nose, throat and ear strains appear to be representatives of the same organism. On agar these have all thick, white sticky growths which appear rather dry on the surface. On serum the growth is usually thicker and even in overnight cultures rather drier and rougher than that of *C. diphtheriae*.

Morphologically in pure culture on serum they are usually of moderate length or short, slender, beaded or striated and not infrequently very deeply stained. They can usually be distinguished from *C. diphtheriae* by the comparative uniformity both of the individual organisms and their arrangement. But in these strains, as in other members of the genus, variations in morphology have been observed from time to time. Though such variations have commonly lessened the resemblance to *C. diphtheriae*, the change at times has been towards a more diphtheria-like appearance. In the thinner parts of such smears the resemblance may be rather striking but even in those cases in the thicker parts the uniformity of arrangement usually gives a clue to the nature of the organism.

The genital strain only differs from those just described in that a yellow pigment is produced.

The distinguishing character of the remaining strain in the group isolated from a whitlow is that on both agar and serum the growth is very fine though the morphology is very similar to that of the other strains in the group. The fact that larger white variant colonies on one occasion appeared on an agar culture of this strain has possibly some bearing on its relation to the other members of the group especially as from time to time these thick growing strains have given thick and thin growths and even occasionally when picked on to agar from plates of Douglas medium growths in which small thin irregular colonies predominated, have been found. Considered by itself this group seems to be a very definite entity but Group III contains several strains in which the growth on agar and serum is indistinguishable from that just described as characteristic of Group V and having somewhat similar morphological characters. Yet these do not ferment galactose thus again emphasising the difficulty of completely correlating biochemical and other characters.

As already indicated, the special interest of the strains in Group V is whether they should be regarded as diphtheroids or as non-virulent strains of *C. diphtheriae*. In this connection it is of interest that all the four strains of this group in Series I had been regarded as diphtheroids on morphological or cultural grounds or both before their examination on the extended series of carbohydrates had shown their biochemical similarity to *C. diphtheriae* and before animal experiments had shown them to be non-virulent. The further study of these and the additional strains of the group in Series II confirms on the whole this opinion though it has shown that owing to occasional morphological and cultural variations there may be a temporary difficulty in placing them with certainty. It may be admitted that there is a certain temptation to build a theory of gradual loss of characters on the part of virulent strains but at present definite evidence in support of this is lacking. Should at any time, however, a virulent strain be found having the thick white sticky growth characteristic of Group V, the position of the strains in that group would at once have to be reconsidered, as owing to possible morphological variations and to the personal element which enters into the morpho-

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logical diagnosis of doubtful strains of corynebacteria the growth on agar must be regarded as the chief point of distinction between this group and non-virulent strains which morphologically and culturally are quite indistinguishable from virulent *C. diphtheriae*.

### *Group Va.*

Two strains both isolated from throat swabs within the last three months have been somewhat tentatively placed in this sub-group. Their biochemical characters differ only from those of the Group V strains in that the maltose reaction is definitely weaker. The latter almost invariably produce acid and clot in this carbohydrate whereas the reaction of those in the sub-group resembles more the incomplete and temporary reaction given by Groups I, II and IV in maltose. In one of the strains the fermentation of galactose is unusually slow. The fermentation reactions are almost the only point these two *Va* strains have in common with each other. The first has a thick yellow growth on agar and on both agar and serum the morphology is very similar to that of the Group V strains. The second strain has a thick moist sticky growth on agar and a very profuse moist growth on serum. Morphologically in pure culture on both media it resembles *C. hofmannii* very much more than *C. diphtheriae* though in the mixed culture from the throat it showed marked polar staining. Both strains like those in Group V are non-virulent.

### *Groups VI and VIII.*

The strains in these two groups are only distinguished from each other by the fermentation of lactose by those in Group VI. As already mentioned (p. 244), this is a true fermentation of that carbohydrate to be distinguished from the temporary reaction given by *C. diphtheriae* and some diphtheroids. In morphology and the cultural characters studied the strains in the two groups are indistinguishable from each other. All have dry wrinkled growths on agar and thick rather dry growths on serum and all produce in some degree a yellow pigment (see p. 250). But the greatest interest of the strains is morphological. With the exception of one strain from the ear all were isolated from throat swabs and in smears from the primary mixed cultures the resemblance of individuals and small groups to *C. diphtheriae*, together with a certain pleomorphism at times gave rise to considerable difficulty in diagnosis. In the Diphtheria Monograph it is pointed out that the possibility of confusion with *C. diphtheriae* disappears in pure culture where they appear as short or more often very short slender rods staining somewhat irregularly or showing well-marked polar granules. Both the individuals themselves and their arrangement in relation to each other present an appearance of uniformity which is very different from that of *C. diphtheriae*. To exclude as far as might be the possibility that the diphtheria like forms seen in the primary cultures really represented *C. diphtheriae* which was subsequently overgrown by the second diphtheroid very exhaustive fishing of the plates was practised. This failed

to yield any evidence of *C. diphtheriae* and though in some cases a diphtheroid of another group was found (p. 248) in only one instance was it present in such small numbers that it would have been at all likely to have escaped observation in the ordinary examination of the plates. A further study of the morphology has also given support to the first opinion that the diphtheria-like forms seen in the primary cultures represent the diphtheroid and not *C. diphtheriae*; for from time to time though by no means constantly a morphology similar to that of the primary cultures has been found in pure culture. This was seen perhaps most frequently in young cultures (4, 6 and 7½ hours growth) but has also appeared in older cultures. Even when present the diphtheria-like forms may be so few as to readily escape notice if not especially looked for and as a rule even in young cultures the predominant forms are those described as characteristic of these groups. On the other hand, occasionally in the same culture diphtheria-like morphology has predominated at one stage of growth and the group morphology at another.

#### Group XI.

It might be supposed that the non-fermenting organisms in Group XI were all representatives of *C. hofmannii*. Though strains of this organism are included other members of the group chiefly on morphological grounds could not be considered to be identical with it. These were often very short and slender, frequently somewhat solid with a rather indefinite median division; less commonly they showed well-marked polar staining. But with these as with other members of the genus the presence of polar granules was a somewhat variable character. The most interesting member of the group was one isolated from the ear of a child in an isolation hospital. It was of moderate length, slender, slightly curved, somewhat solid perhaps but often tapering off at one end and showing a polar granule at the other. The appearance suggested a young culture of *C. diphtheriae* but it ferments no carbohydrates and is non-virulent. It is probably an example of the *C. ceruminis* of other writers. Another strain in the group of nasal origin when first isolated was morphologically indistinguishable from *C. diphtheriae* though its morphology was of a different type from that just described. In some sub-cultures, however, the resemblance has been much less marked. Three strains in the group were distinguished from the others by the production of pigment. In two of these isolated from the nose the colour was a deep orange, in the third of genital origin it was a bright yellow.

The solitary strain in Group IX isolated from an oyster differs from all the other diphtheroids in the series in liquefying serum.

Mannite did not prove a very useful differential substance in the first series and was not used in the second. It was originally included because the great majority of previous workers who had used it in the examination of corynebacteria agreed that it was not acted upon by *C. diphtheriae*; whilst Douglas and his co-workers in their investigation of wound diphtheroids had

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found a mannite fermenting group. In Series I mannite was only attacked by one strain (Group X). The reaction was slow, not appearing until 4th or 5th day and though definite was sometimes incomplete.

### DIFFERENTIATION OF *C. DIPHTHERIAE* FROM THE DIPHTHEROIDS BY MEANS OF FERMENTATION REACTIONS.

It will be noticed that Table II has been divided into three sections. Section A contains the groups which ferment some carbohydrates but not cane-sugar, B those fermenting cane-sugar and C the non-fermenters. This division of the groups is of interest because of its bearing on the possibility of separating virulent *C. diphtheriae* from all other members of the genus by means of fermentation tests. The existence of a number of strains, regarded as non-virulent *C. diphtheriae* which cannot be distinguished from *C. diphtheriae* either by biochemical reactions, morphology or other characters has already been alluded to. On the other hand, the fermentation of cane-sugar or the absence of fermentation of both glucose and cane-sugar have long been regarded as points of distinction between the diphtheroids and *C. diphtheriae*.

Of the 150 diphtheroids examined in the course of this work, 122 of which came from the ear, nose and throat, 71 per cent. from all sources or 74 per cent. if ear, nose and throat strains only are considered came into this category. Of the remainder those in Groups V and Va have the same fermentation reactions as *C. diphtheriae* but those in Groups III and IV 28 (18.6 per cent.) in all, of which 19 (15.5 per cent.) are from ear, nose and throat, do not ferment galactose and so can be distinguished from *C. diphtheriae* by that means. This seems to be a point of some practical importance particularly in laboratories having no facilities for animal experiments. Most of the strains in these two groups were isolated because of doubtful morphology in the primary mixed cultures. It is true that in *pure* culture many of them can be distinguished with comparative certainty from *C. diphtheriae* on morphological grounds. On the other hand, it is well known that corynebacteria are subject to considerable variations in morphology and that in doubtful cases the personal element enters largely into such diagnoses. In these circumstances any additional point of distinction would appear to be of considerable value.

The position of groups V and Va has already been very fully discussed (p. 254) but for reasons similar to those just advanced in favour of the use of galactose in the differentiation of Groups III and IV it is probably advisable in practice to regard all organisms fermenting glucose and galactose and not attacking cane-sugar as possible *C. diphtheriae* and to test their virulence even though the morphology and cultural characters of a few strains may justify an experienced bacteriologist in classifying them as diphtheroids.

## SUMMARY AND CONCLUSIONS.

The fermentation reactions and some ancillary characters of 102 strains of virulent *C. diphtheriae*, 31 non-virulent strains and 150 diphtheroids have been studied.

With the possible exception of dextrin which proved to be an unsuitable substance for biological tests all the strains of virulent *C. diphtheriae* examined were found to have constant biochemical reactions, fermenting glucose, maltose and galactose and having no action on cane-sugar, lactose and mannite.

The strains classified as non-virulent *C. diphtheriae* had the same biochemical characters as the virulent strains and were indistinguishable from them either on morphological or cultural grounds.

The diphtheroids examined showed great diversity in their power of attacking the carbohydrates used, at least eleven biochemical groups being recognised.

By the use of three carbohydrates, glucose, galactose and cane-sugar 90 per cent. of the diphtheroids in this series could be distinguished from *C. diphtheriae* by their fermentation reactions. In the remaining 10 per cent. a clue as to the nature of the organism is often obtained from the character of the growth on agar.

Among the corynebacteria examined other than *C. diphtheriae* no constant relationship was found between source, biochemical, morphological and other characters.

Nevertheless in dealing with members of the genus in pure culture, the morphology, character of the growth on agar and fermentation reactions taken together give useful information as to whether the organism under consideration is *C. diphtheriae* (virulent or non-virulent) or a diphtheroid.

My best thanks are due to Dr Ledingham for much help and advice given throughout the work and to Dr Atkin and Dr Robison for their assistance in dealing with the lactose and dextrin problems respectively.

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and five months later were found to be free from illness; two other animals inoculated with the gland plus lactic acid, developed tubercular disease.

*Remarks.* The experiment appears to show the sensitising action of the lactic acid.

*Case 10.* Only one guinea-pig was inoculated with the gland alone, and no infection resulted. Of the three animals which received the gland plus lactic acid, one remained healthy, one showed isolated liver and lung nodules, and one marked tuberculosis of the liver.

*Remarks.* Again in favour of the sensitising action of the lactic acid, although the use of only one control renders the experiment of little value.

In order that Fraenkel and Much's results as a whole can be seen at a glance, their animal experiments have been tabulated (see Table I).

Table I.

Case	Inoculations	Material	Died early	Nil	T.B.	? Lymphad.
1	4	Gl	2	.	2	.
	4	L	.	4	.	.
2	4	Gl	.	? 4	.	.
	4	L	.	.	4	.
	6	T.B.	.	.	6	.
	6	" L	.	.	6	.
	6	" A	.	6	.	.
3	8	A	.	8	.	.
	8	AL	.	8	.	.
	8	Gl	4	2	.	2
	8	L	2	3	2	1
4	?	.	.	.	.	.
5	2	Gl	.	2	.	.
	4	L	.	3	1	.
6	3	A.-m. Gl	.	3	.	.
	3	" L	.	3	.	.
	2	" A	.	2	.	.
	2	" AL	.	2	.	.
	1	P.-m. Gl	.	.	1	.
	2	" L	.	.	2	.
7	2	Gl	.	1	1	.
	4	L	.	3	1	.
8	?	.	.	.	.	.
9	2	Gl	.	2	.	.
	2	L	.	.	2	.
10	1	Gl	.	1	.	.
	3	L	.	1	2	.

Gl=Untreated gland. L=Gland treated with lactic acid. A=Gland treated with antiformin.

The conclusions that these authors draw from their experiments will now be given, together with my own criticism of their conclusions:

1. Lymphadenoma is a rare form of tubercular disease, but not rarer than some other forms of tubercular disease (for example, *Lupus erythematosus*).

*Remarks.* Lymphadenoma may be a rare form of tubercular disease, but I am of the opinion that the experimental proof offered is by no means sufficient to render such a conclusion justifiable. Tubercle bacilli were isolated on eight occasions from 11 specimens obtained from 10 cases. From four

cases only were tubercle bacilli isolated from some of the animals inoculated with the tissue plus lactic acid, while the control animals did not develop tubercular disease. But the number of control animals was in three out of these four cases not as great as was that of the animals inoculated with the gland plus lactic acid.

2. Special constitutional changes are necessary for its genesis. It is impossible to say what the changes are, as it is equally impossible to say the conditions governing the development of other tubercular diseases such as *Lupus vulgaris*, *Lupus erythematosus* or *Erythema induratum*.

*Remarks.* I agree with the authors that special constitutional changes are probably necessary for its genesis.

3. The fact that Lymphadenoma cannot be transmitted to animals is no argument against its being tubercular in nature, for the three diseases mentioned in the previous paragraph cannot be given to animals, and yet their tubercular nature is established with certainty.

*Remarks.* The fact that Lymphadenoma cannot be transmitted to animals is no argument against its being tubercular, but is it established beyond doubt that *Lupus erythematosus* is tubercular in nature? As regards *Lupus vulgaris*, the histological picture contains many of the characteristics of a tubercular process, and a positive reaction is usually obtained with most of the tuberculins.

4. Changes have been produced in animals which are indistinguishable from those found in Lymphadenoma; but much value is not placed on such experiments seeing that Baumgarten and Lichtenstein produced similar changes by the inoculation of small doses of ordinary tubercle bacilli.

*Remarks.* The authors' description of the histological lesions they found in some of their animals are not convincing. Also, in the light of my own animal experiments with acid-fast bacilli, I find it impossible to accept unreservedly the isolated experiments of the workers who claim to have produced typical Lymphadenoma in animals by the injection of small quantities of tubercle bacilli.

5. The body of the host weakens the bacillus, which is evidenced by: 1st, the fact that antiformin destroys the bacilli, while ordinary tubercle bacilli are not killed; 2nd, that the virulence can be heightened by the use of such a reagent as lactic acid; and 3rd, that on taking a series of animals, only certain members become tubercular.

*Remarks.* Here one may ask why the antiformin did not destroy the infective agent in the 1913 experiments. Also, the case 2 experiments seem to point to the possibility of the strength of the antiformin being rather weak. As regards the sensitising action of the lactic acid, cases 1, 2, 4 and 8 must obviously be left out of account. We then find that of the 15 animals inoculated with the gland alone, 4 developed disease and 11 remained healthy; of the 24 animals inoculated with the gland plus lactic acid, 11 became diseased and 13 remained healthy: thus 26.6 and 45.83 per cent. respectively of the animals inoculated became diseased. When the gland alone was used



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there were only two cases of irregularity, where only some of the inoculated animals developed disease; with the lactic acid animals there were four cases of irregularity.

The animal experiments carried out in these laboratories, in an endeavour to confirm the results of the German workers, will now be briefly discussed.

### *Comparative animal experiments performed in these laboratories.*

Specimens from 44 cases which were suspected clinically of being Lymphadenoma have been examined. On microscopical examination, 27 were found to be certainly lymphadenomatous, containing the typical karyokinetic giant cells and numerous eosinophile cells, etc.; three were probably lymphadenomatous, containing eosinophile cells, etc. but not the typical giant cells; seven showed a picture of chronic inflammatory changes, and were possibly lymphadenomatous; four were tubercular in nature; three were sarcomatous; one was later diagnosed as glandular fever, but the patient has since died of phthisis; and one which was thought to be an acute case of Lymphadenoma, only showed an acute periportal inflammatory condition of the liver. In three of the lymphadenomatous cases histological evidence of tubercular disease was also found, on the one hand the two diseases being intimately mixed with one another in several organs, while in one case lymphatic glands which appeared to be purely lymphadenomatous were associated with tubercle of the liver and lungs. On one or two occasions where ante-mortem specimens from a patient have been typical, post-mortem specimens from the same patient have been doubtful, and *vice versa*.

Of the 24 cases of Lymphadenoma, which were unaccompanied by tubercular disease, only 16 fresh specimens suitable for animal inoculations were obtained.

In Table II a list is given of the guinea-pigs inoculated with tissues obtained in the fresh state from the 16 certain cases of Lymphadenoma.

Cases 6, 14 and 29 showed a picture of chronic inflammation, case 16 of acute inflammation, case 19 was later diagnosed as glandular fever, and case 43 turned out to be a Lymphosarcoma. Case 34 showed histologically a picture of an old tubercular gland, with much caseation and calcification, the parasite having evidently been destroyed. None of the 94 animals became tubercular, and it will be noted that on 26 occasions the inoculum was treated with varying dilutions of lactic acid. Again, none of the 16 animals mentioned in Table III became tubercular, they being injected with material, probably lymphadenomatous; and the inoculum was treated with lactic acid on seven occasions. The 26 animals mentioned in Table IV also remained healthy, on nine occasions the inoculum being treated with lactic acid.

Thus we find that not one of the 136 animals inoculated with tissues obtained from suspected cases of Lymphadenoma developed tubercular disease, although on 42 occasions the inoculum was injected with lactic acid. It may be mentioned that a considerable number of animals have died at an early

date from diseases other than tuberculosis, all such animals being excluded from the above totals; only those which survived for at least six weeks after the injection being taken into account.

Table II. *Guinea-pigs inoculated with glands, etc., received in the fresh state, from typical cases of Lymphadenoma.*

Case	3.	2 guinea-pigs, with	fresh gland
	6	"	" glycerinated gland
"	7.	2	" " spleen
"	8.	3	" " fresh gland
	6	"	" gland kept in broth
"	9.	3	" " saline
	3	"	" " broth
	7	"	" glycerinated gland
"	12.	2	" " fresh gland
"	21.	2	" " "
"	23.	2	" " glycerinated gland
"	24.	1	" " "
"	26.	3	" " fresh gland
"	27.	1	" " blood
	1	"	" gland kept in broth
	1	"	" bone marrow kept in broth
	1	"	" gland plus lactic acid
"	28.	2	" " gland kept in broth
	2	"	" " plus lactic acid
"	30.	1	" " fresh gland
	3	"	" " gland plus lactic acid
"	31.	1	" " " kept in broth
	3	"	" " plus lactic acid
"	36.	6	" " fresh gland
	6	"	" " gland plus lactic acid
"	40.	6	" " fresh gland
	6	"	" " gland plus lactic acid
"	41.	6	" " fresh gland
	6	"	" " gland plus lactic acid
Total	94		

Table III. *Guinea-pigs inoculated with gland, received in the fresh state, from cases probably lymphadenomatous.*

Case	22.	3 guinea-pigs, with	fresh gland
	1	"	" gland kept in broth
	2	"	" " plus lactic acid
"	42.	3	" " fresh gland
	3	"	" " gland plus lactic acid
"	44.	2	" " fresh gland
	2	"	" " gland plus lactic acid
Total	16		

Table IV. *Material from cases diagnosed clinically as Lymphadenoma, but in which histologically there was no specific evidence of Lymphadenoma.*

Case	6.	6 guinea-pigs, with	gland kept in saline
"	14.	1	" " fresh gland
	1	"	" " gland plus lactic acid
"	16.	1	" " fresh gland
"	19.	1	" " "
"	29.	1	" " "
	2	"	" " gland plus lactic acid
"	34.	6	" " fresh gland
	6	"	" " gland plus lactic acid
"	43.	1	" " fresh gland
Total	26		

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It is very difficult to account for the striking difference between these figures and those obtained by Fraenkel and Much, not only in so far as the lactic acid experiments are concerned, but also as regards the controls. Among their 16 control animals which can with safety be considered, six developed disease, either ordinary tubercular disease or what the authors call a condition resembling Lymphadenoma. My 39 animals injected with untreated gland all remained healthy. Where lactic acid was used, 11 out of their 21 animals became diseased; again my 42 animals all remained healthy. As a matter of fact the 55 controls inoculated with material kept in saline, broth and glycerine are also of value for it has been found that tubercle bacilli kept in such liquids, in cold storage, survive for many months; the number of controls then becomes 94, all of which survived.

The injections made by Fraenkel and Much were with massive doses of the inoculum, and, in their opinion, this is one of the reasons why they were so successful in isolating the tubercle bacillus from such a number of cases. Many of my injections were, however, massive, and, what is more, many of them were repeated on several occasions. Frequently solid lumps of tissue were injected subcutaneously, which presumably would favour the development of any rare or fragile bacillus which might be present, but there was on no occasion resulting tubercular disease. The weights of my animals have been taken every week during the early stages of the experiments, in order, if possible, to register any transitory illness as a result of the injections. Provided the material inoculated has been free from ordinary bacterial contaminations, I have obtained no reliable evidence whatsoever that the injections affect materially the general health of the animals.

I can find no mention by Fraenkel and Much of control animal experiments with other lymphatic glands such as those obtained from cases of leucaemia or pernicious anaemia; it would be interesting to know what proportion of such diseased glands would, in their hands, infect the guinea-pig with tubercular disease.

In these laboratories the organs from about 20 cases of leucaemia and pernicious anaemia have been examined, and where there has been no histological evidence of tubercular disease, pieces of the tissues have been inoculated into animals. Some 50 guinea-pigs have been so inoculated, and six animals only developed tubercular disease among 12 inoculated with tissues derived from a single case of myelocytic leucaemia. None of the tissues were treated with lactic acid, for such control experiments, in these laboratories, were not considered necessary seeing that attempts to produce tubercular disease with lymphadenomatous glands failed.

Attempts were made to heighten the virulence of certain acid-fast bacilli by treating them with lymphadenomatous tissues and their extracts, and by simultaneous injection of the bacilli and the diseased tissues into animals. Saprophytic acid-fast bacilli were mostly used, and especially *B. phlei*. The last mentioned bacillus I consider to be only slightly less pathogenic for the

rabbit than the human tubercle bacillus, although with an equal area of diseased organs the wasting of the animal shows the greater toxicity of the tubercle bacillus. So far one has not been able to detect any special effect of lymphadenomatous tissues or their extracts on the virulence of the bacilli tested; and similar negative results were obtained when using carcinomatous and leucaemic tissues. It has been observed that the repeated injection of tubercular lymphadenomatous glands into the peritoneal cavity of guinea-pigs leads to death of the animals at a very early date, but one would imagine that glands from cases of other diseases secondarily infected with tubercle bacilli would similarly kill rapidly if injected repeatedly.

The lymphatic glands from a case of Lymphadenoma in which the liver and lungs were tubercular while the spleen and lymphatic glands appeared microscopically to be lymphadenomatous only, caused death of the six guinea-pigs inoculated within a few weeks. The histological picture in the organs of these animals was not that of typical tubercular disease, several massive injections of cell emulsions having been injected. The description given by Fraenkel and Much of the microscopical findings in some of their animals seems to correspond pretty well with that found in these six animals—giant cells containing several nuclei (not of Langhans' type) with fairly active mitosis and large areas of fibrosis practically free from cellular elements were present; but acid-fast bacilli were found, and the general appearance was not that of a lymphadenomatous organ.

It has been found that the presence of lymphadenomatous tissues or their extracts in fluid culture media has no noticeable effect on the rapidity or extent of the surface growth of tubercle bacilli and allied saprophytes.

#### CONCLUSIONS.

The evidence at present available in support of the view that Lymphadenoma is a special manifestation of tubercular disease is not conclusive: Lymphadenoma glands may often be infected with tubercle bacilli, but possibly not more often than they are infected with streptococci or diphtheroids.

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## TWO CASES OF MELIOIDOSIS.

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WE have described under the name Melioidosis a fatal disease affecting man and rodents, the latter constituting in all probability a natural reservoir of the virus (Stanton and Fletcher, 1921). Whitmore (1913) first drew attention to this malady as "a glanders-like disease occurring in Rangoon" and isolated the organism, now known by his name, which is the cause of the disease. It is a small Gram-negative bacillus which sometimes bears a strong resemblance to the *Bacillus pestis*, particularly in early subcultures and in films prepared direct from lesions in the body where as a rule it is very scanty. The lesions themselves, as emphasised by Whitmore, are very like those of glanders; but *B. whitmori* possesses two attributes which distinguish it at once both from plague and from glanders bacilli: it is actively motile and it grows on glycerine agar in a most striking and characteristic manner, forming curious wrinkled, corrugated colonies. There is also a mucoid form, analogous with the mucoid variety of *B. paratyphosus* B. Both forms are shown in Fig. 1.

The majority of the cases which have been studied hitherto have not been examined by a medical man until after death, and we are reporting these two because, in the first place, each of them had been in hospital for several days before death took place and, secondly, because one of them is remarkable in being the only instance of the disease which has been recognised in a European.

*Case 1.*

THE FIRST PATIENT was a robust Tamil cooly, forty years of age, who was admitted to the District Hospital at Kuala Lumpur on November 7, 1923, complaining of fever, which had commenced twelve days previously, and pains in the epigastrium. During the time he was in hospital, his temperature was irregular and ranged from just above normal to 102° F. No malaria

parasites were found in his blood and no physical signs of disease could be detected, except a tender and greatly enlarged spleen. Five days after his admission, the bases of both lungs were consolidated and, whereas he was constipated when he came into the hospital, he now began to have diarrhoea. He became restless with hurried breathing, and collapsed on the night of November 14. He died on the following morning, about three weeks after the beginning of his illness.

A POST-MORTEM EXAMINATION was made within a few hours of death and lesions were found in the lungs, liver, spleen and large intestine. There were several areas of consolidation in both LUNGS, two in the left and ten in the right, situated chiefly at the periphery and free edges of the lobes (see Fig. 2). The areas of consolidation were from 1 to 2 cm. in diameter, but in some places they had coalesced to form larger masses. Projecting beneath the pleura, they gave a knobbed appearance to the surface of the lungs. On cutting through one of the larger patches, it was seen that there ran through the centre of it a blood vessel bearing upon its branches, like fruit upon the branches of a tree, minute tubercle-like nodules which had coalesced to form a larger area of consolidation. There was no inflammation of the pleura overlying these areas, which were composed of aggregations of small suppurating points, the tubercle-like nodules just mentioned. In films made from the lungs there were many minute, slender Gram-negative bacilli, most of them extra-cellular, and also a few Gram-positive cocci. No organisms were seen which bore any strong morphological resemblance to *B. whitmori*, but this bacillus grew in cultures made from the patches of consolidation.

The LIVER was enlarged and weighed 1900 grms. (the average weight, in Tamils, is 1372 grms.). There were five rounded, projecting bosses on its surface; the two largest measured about 4 cm. in diameter and contained yellow pus which could be seen through Glisson's capsule. When the organ was incised the smaller lesions were found to consist of aggregations of minute suppurating tubercles which, in the two larger ones, had coalesced to form sharply circumscribed abscesses of a honeycomb character. The lesions were in the periphery of the organ and were apparently the result of septic infarction. The GALL-BLADDER was thickened and the mucous lining inflamed (see Fig. 3). Films prepared from the pus in the liver contained some Gram-positive diplococci, like pneumococci, and also a few Gram-negative bacilli which stained in a bipolar fashion with Leishman's stain. *Bacillus whitmori* was cultivated from the liver pus and from the gall-bladder.

The SPLEEN (see Fig. 4) weighed 1450 grms., or nearly three times its normal weight. In it, there were numerous septic infarcts some of which were suppurating, and one measured  $4 \times 1.5$  cm. in diameter. The capsule was greatly thickened. A few very small, slender, Gram-negative bacilli, and some Gram-positive diplococci were seen in films prepared from the spleen. *B. whitmori* was cultivated from the pus in the abscesses.

The gastro-intestinal canal was apparently healthy, except the CAECUM,

in which there were several small erosions, each covered with a granular layer of adherent debris (see Fig. 5). In one spot there was a definite ulcer, about 1 cm. long, with overhanging edges. Cultures were made from this ulcer and also from the erosions, but *B. whitmori* was not isolated.

Further investigations were made into the characters of the strains of *B. whitmori* cultivated from the lungs, spleen, liver and gall-bladder. These cultures resembled the laboratory type strain ("*Ragaviah*") of the organism in growth and morphology, in their action on sugar, in their liquefaction of gelatine and in all other respects. They were agglutinated to full titre by an immune serum prepared with the type strain of *B. whitmori* but not by a *B. mallei* serum prepared with the Lister Institute strain Minett.

Several ANIMALS WERE INOCULATED, some with pus from the abscesses and others with cultures. Two GUINEA-PIGS, *A* and *B*, were inoculated with pus from the liver. A little of the pus was introduced into the right nostril of guinea-pig *A*, and six days later, the dirty condition of the nose showed that there was some discharge. On the eighth day the right eye was inflamed and, on the following morning, the animal died. Post-mortem, the subcutaneous tissue was red and injected, and there was an enlarged caseous gland in the right inguinal region. The nose was red and swollen, and inside it there was a little thick pus. *B. whitmori* was recovered in almost pure culture from the nose and from the gland.

Guinea-pig *B* was inoculated under the skin of the abdomen with the same pus, and at the same time, as guinea-pig *A*. A small hard, red swelling resulted, and a little ulcer appeared at the point of inoculation. On the ninth day, by which time guinea-pig *A* was dead, the ulcer had healed and the swelling had almost disappeared. The animal was lively and was eating well; but there was an enlarged gland in the right groin. This gland suppurated and other buboes appeared on the shoulders and in the axillae. By the end of the third week the guinea-pig was not eating well and it died on the twenty-fifth day after inoculation. At the autopsy, abscesses were found in the groins and axillae of both sides, and also a large one, full of pus which resembled curdled milk, in the region of Douglas's pouch. *B. whitmori* was recovered in pure culture from these abscesses. A third guinea-pig was inoculated subcutaneously with a culture from the animal and died within sixteen hours from septicaemia due to *B. whitmori*. Post-mortem, there were enlarged haemorrhagic inguinal glands, a slightly enlarged spleen, and subcutaneous haemorrhages, such as are seen in a guinea-pig that has died from plague. *B. whitmori* was cultivated from the heart and spleen.

Another guinea-pig, *D*, was inoculated subcutaneously with the same strain, which had been grown for forty-eight hours in 10 c.c. of broth to which 0.25 c.c. of a high titre immune serum had been added. A small abscess developed at the point of inoculation, which ruptured and discharged its contents. The guinea-pig continued active and ate its food; but the axillary, subscapular and inguinal glands became enlarged and it died on the twenty-fifth

day, with suppurating buboes containing thick creamy pus, and with small abscesses in the liver. The spleen was enlarged, the left lung was hepatised and the right lung and pleura were filled with suppurating tubercles. *B. whitmorei* was cultivated from the buboes and from the viscera. Several other guinea-pigs were inoculated with results similar to those we have already described.

In order to determine if HORSES are susceptible to melioidosis, which resembles glanders in so many ways, an Australian mare was inoculated subcutaneously by Major Symonds, Veterinary Surgeon F.M.S., with about five hundred million organisms of the fourth subculture on agar. The mare's health did not appear to be disturbed, nor was there any rise of temperature or general reaction. A small fluctuating swelling, half the size of a walnut, appeared at the point of inoculation, but it subsided in a week, and when the mare was killed, four months later, there were no signs of melioidosis, nor could Whitmore's bacillus be cultivated from the blood or viscera.

A Java pony, inoculated in the same way, by Major Symonds, at the same time as the mare, developed a tender swelling and a slight rise of temperature. There was a little fever for ten days and the swelling suppurated; but, by the end of three weeks, the resulting ulcer had healed and the pony seemed none the worse. She was killed four months later and no signs of infection could be discovered.

### Case 2.

THE SECOND PATIENT was a European, forty-two years of age, who was admitted to hospital on December 17, 1923, with diarrhoea and malaise which had lasted for ten days. He complained of pain in the epigastrium which came on almost immediately after food, and on five occasions he had vomited. He had been in the Malay States for sixteen years, and for three months prior to his illness he had been living in a mining village, in a house said to be infested with rats. We wish to draw attention to this, because melioidosis is a disease which occurs naturally in rodents.

We are indebted to Dr J. G. Castellain, the Medical Officer in charge of the European Hospital at Kuala Lumpur, for the clinical notes. The patient's temperature was 99.4° on admission, and it swung between subnormal and 102° for the first five days, after which it never sank below a hundred degrees. It rose to 106.4° just before his death, which took place fourteen days after his admission and twenty-four after he began to feel ill. While he was in hospital, he complained of pain in the pit of the stomach, where there was considerable tenderness, and the liver was definitely enlarged. He vomited on several occasions and, with the swinging of his temperature, he had rigors and sweats. During the first few days in hospital he was passing six or eight loose motions daily but this diarrhoea improved and, for the last week, his bowels acted not more often than two or three times in twenty-four hours. His stools contained no blood or mucus, nor were amoebae or dysentery bacilli found on examination. No malaria parasites were seen in his blood;



the proportion of polynuclear leucocytes was not increased. The patient became progressively weaker, sank into a low muttering delirium and died.

A limited POST-MORTEM EXAMINATION was made within a few hours of death. The LUNGS and SPLEEN appeared to be healthy on superficial examination, but they were not incised. The peritoneum was apparently normal except for some adhesions of the omentum to the surface of the liver.

On the upper surface of the left lobe of the LIVER there was a dark congested swelling with yellow points of pus showing through Glisson's capsule. There was a similar, though smaller, swelling in the middle of the upper surface of the right lobe. Each of these tumours was a large abscess. That on the left side was spherical and about 6 cm. in diameter. It contained only a few ounces of thick yellow pus. The wall consisted of a layer of shaggy necrotic material about 2 cm. in thickness. The abscess in the right lobe was a little smaller, but two finger-like projections extended from it. One towards the quadrate lobe and the other upwards. There was very little pus in this second abscess. Films prepared from scrapings of the abscess wall consisted of necrotic cells, most of which contained fat globules. There were also a few red blood corpuscles, but no healthy polynuclear pus cells. No amoebae could be found although a search for them was made in several films. No micro-organisms were seen in stained films, but *B. whitmori* grew on glycerine agar inoculated with the material.

The GALL-BLADDER was at least an eighth of an inch thick and resembled the gall-bladder of the other patient, Velu. Unfortunately no cultures were made from it.

In the large intestine there were no amoebic lesions, but there were three or four small ulcers, in the CAECUM, which bore some resemblance to those found in a similar position at the post-mortem examination of Velu.

The BACTERIOLOGICAL INVESTIGATION of this case was incomplete. At the autopsy we jumped to the hasty conclusion that the abscesses were associated with *E. histolytica*, and nothing but a portion of the wall of the abscess in the right lobe of the liver (see Fig. 6) was taken to the laboratory for further investigation. Glycerine agar slopes were inoculated with material from this and, on these, *B. whitmori* grew in almost pure culture. The colonies assumed their usual form within forty-eight hours, a raised dome in the centre with a wide flat margin around it, like the broad-brimmed straw hats worn by Chinese in ceremonial processions. After two or three days the growth became characteristically corrugated. Individually, the bacilli were actively motile and, in the earlier subcultures, they showed excellent bipolar staining with Leishman's stain. The organisms grew rapidly in peptone water, causing great turbidity and forming a wrinkled pellicle on the surface of the medium. Acid, but not gas, was produced in glucose and saccharose, within twenty-four hours. Lactose was fermented on the fourth day and also mannite and dulcitol at the surface of the medium, where they were in contact with the air. In later subcultures the power of fermenting sugars was considerably less.

Gelatine was liquefied on the fourth day. The organism was agglutinated to full titre by an immune serum prepared from the type laboratory strain (Ragaviah), and to half titre by an old sample of blood serum collected from the patient from whom this type strain was isolated.

Several ANIMALS WERE INOCULATED with the organism isolated from this case. Two wild RATS (*Mus griseiventer*, Bonhote) were inoculated subcutaneously with about a thousand million organisms, grown for twenty-four hours on an agar slope. In these animals the disease, following inoculation or feeding with contaminated food, usually runs a chronic course lasting about three months, but both these rats died from acute septicaemia in less than twenty-four hours, with great subcutaneous redness and congestion of all the organs. *B. whitmori* was cultivated from the heart's blood of both the rats, although—as is usually the case in infection with this bacillus—none could be found in films made directly from the blood.

TWO GUINEA-PIGS were inoculated at the same time as the rats; one subcutaneously with a thousand million organisms, the other by the introduction of a loopful of the culture into the left nostril. Both animals died from septicaemia. The first one died forty-eight hours after inoculation; with redness and oedema at the site of injection, redness of the subcutaneous tissue and enlarged haemorrhagic inguinal glands. The second animal died on the fifth day, with the usual signs of septicaemia and a cheesy deposit in the left nostril, which contained *B. whitmori*. This organism was cultivated from the heart in both cases. Five weeks later, a third guinea-pig was inoculated in the left nostril with the bacillus which had been subcultured repeatedly on glycerine agar, and in this case the disease ran a more chronic course. Slight discharge from the left side of the nose and from the left eye was noticed on the third day. The animal became thin, refused its food, and died on the twelfth day with enlarged caseous glands on each side of the trachea. *B. whitmori* was recovered from the glands and from the spleen.

A RABBIT, weighing 1300 grms., was inoculated under the skin of the abdomen with about a hundred million organisms. It died in thirty-six hours. Post-mortem, it was seen that the inflammatory process, set up by the injection of the virus, had extended inwards from the point of inoculation, through the abdominal wall to the peritoneum. There were minute tubercles on the parietal peritoneum, scattered along the veins and forming a definite cord along the lymphatics leading from the spot where the injection had been made. The under surface of the diaphragm was thickly covered with minute tubercles and the general appearance was very like that of miliary tuberculosis. *B. whitmori* was cultivated from the heart, spleen and local lesions.

MONKEYS are susceptible to melioidosis and have been infected by feeding. Half a cubic centimetre of the second subculture of *B. whitmori* from this case, containing about five hundred million organisms, was squirted into the mouth of a *Macacus cynomolgus* with a pipette. It showed no signs of infection and is apparently in good health, two months later.

A PONY, the same which had been inoculated with the strain of *B. whitmori* from the first patient, Velu, was given an intravenous injection of about a thousand million organisms of the culture obtained from the second, the European case, by Major Symonds. The temperature rose to 103.6° in three hours, and for six days it remained high, but by the end of a week the pony appeared to have recovered. It was killed sixteen weeks later and at the post-mortem examination no signs of melioidosis were discovered.

The first point to be noticed in a DISCUSSION of this report is that in neither case was the nature of the disease discovered during the life of the patient. Indeed, this has been done in only four out of the forty-eight cases in which melioidosis has been recognised. In one instance *B. whitmori* was cultivated from the blood, in another from the urine; in a third, from a pustular eruption, and in the fourth from an abscess. In this last case the suspicions, aroused by the "positive" result of an agglutination test, were confirmed by the cultivation of the bacillus from an abscess, which appeared later, connected with the lower end of the fibula.

As in animals, so in man, the patient may die from a fulminating, acute septicaemia, like the rats inoculated with the culture isolated from the second case in this report; or the disease may run a more chronic course, as in the guinea-pigs *B* and *D*, which were infected with the pus from the first case. The signs and symptoms of the chronic form are dependent upon the distribution of the lesions. We have seen one patient in which the brunt of the disease fell upon the urinary system and, here, the prominent symptoms were due to lesions of the kidney, ureters and bladder. In the majority of the cases, which have come to our notice, the illness commenced as an acute septicaemia; if the patient did not succumb within the first few days, he passed into a typhoid condition of chronic septicaemia, with the formation of abscesses in different organs. Every case has died in less than four weeks except two; one of which recovered after a long illness, while the other is still an invalid with abscesses, sinuses and chronic suppuration in the bones of his feet and legs, more than two years after the commencement of the malady.

In the two patients which form the subject of this report, the onset of the disease was gradual, whereas, in the other cases which we have encountered, the illness has been characterised by a sudden onset accompanied by collapse and, sometimes, by a purging so violent as to suggest the diagnosis of cholera.

There are no definite clinical signs by which the disease can be recognised: a sure diagnosis can be made only by the cultivation of the causative organism. A "positive" agglutination reaction is of value as presumptive evidence particularly if the dilution increases after an interval (as it did in the instance which we have mentioned, where it rose from 1 in 80 to 1 in 1200 after an interval of three weeks): but this reaction is, obviously, of little value in the ordinary acute form of the disease. The organism has been cultivated from the blood and the urine of human cases and also from pustules and abscesses when these

have been present. In one instance it was cultivated from the faeces of a rabbit. Where the lungs are involved, as they usually are, the injection into a guinea-pig of sputum or of material obtained by puncture of the lung, might make the diagnosis clear. In cases of obscure fever associated with enlargement of the liver, this viscus should be explored with a needle, and glycerine agar slopes and animals should be inoculated with the material obtained.

Whenever an abscess of the liver is aspirated or drained, glycerine agar slopes and guinea-pigs should be inoculated with the pus. We consider it probable that some of the cases, in Malaya at least, which are diagnosed as amoebic abscesses, are really instances of melioidosis.

When we come to deal with the epidemiology of the disease, and attempt to find an answer to the question of the manner in which these two men become infected, we must confess ourselves still in difficulty. Melioidosis can be conveyed to rodents by subcutaneous inoculation, by introducing the virus into the nostrils or by feeding. Subcutaneous inoculation is followed, in animals, by a local lesion before generalisation takes place; but there was no history of any such manifestation in our two patients. Infection by inhalation is unlikely except where a number of cases are closely associated. We are of opinion that the disease was conveyed to these men by the ingestion of food which had been contaminated by rodents infected with melioidosis.

#### SUMMARY.

Two cases of fatal melioidosis are described. The first a robust Indian labourer; the second a well-nourished, muscular European.

*Case 1.* Gradual onset. Irregular fever. Pain in the epigastrium. No physical signs of disease at first, except enlarged spleen; later, there were signs of consolidation at the bases of both lungs. Constipated at first; diarrhoea came on towards the end and the patient collapsed and died at the end of the third week.

At the autopsy; there were small patches of consolidation in the lungs, composed of minute suppurating tubercles. In the liver there were similar aggregations of small suppurating tubercles and two circumscribed abscesses. The gall-bladder was thickened. The spleen was enlarged and contained septic infarcts and abscesses. In the caecum there were small superficial ulcers. *B. whitmori* was cultivated from the lungs, spleen, liver and gall-bladder.

Animals were inoculated as follows: A guinea-pig, inoculated in the anterior nares with pus from the liver, died nine days later with caseous inflammation of the nasal passages and a caseous inguinal gland from which *B. whitmori* was recovered. A guinea-pig, inoculated subcutaneously with pus from the liver, died on the twenty-fifth day with abscesses in the lymphatic glands from which *B. whitmori* was cultivated. A guinea-pig, inoculated subcutaneously with a culture of the organism, died within sixteen hours from septicaemia. *B. whitmori* was recovered from the heart and spleen. A guinea-pig, inoculated subcutaneously with an attenuated culture, died on the twenty-fifth

day with suppurating buboes and with abscesses in the liver and lungs from which *B. whitmori* was cultivated. Two horses were inoculated subcutaneously with cultures of the strain of *B. whitmori* cultivated from the patient. An abscess developed at the site of inoculation, in each case, but the local lesion healed and the animals have, so far, remained healthy.

*Case 2.* The illness commenced gradually with pain after food, occasional vomiting and looseness of the bowels. The temperature became high and of the swinging type, accompanied by rigors and sweats. The liver was enlarged. The patient died on the twenty-fifth day.

At the autopsy, two large abscesses were found in the liver, the gall-bladder was thickened and there were a few small superficial ulcers in the caecum. *B. whitmori* was cultivated from the liver abscess. No amoebae were found.

The following animals were inoculated. Two wild rats were inoculated subcutaneously with a culture from this case and both died from septicaemia in less than twenty-four hours. *B. whitmori* was recovered from the heart's blood. The result of the subcutaneous inoculation of a guinea-pig was the same. A guinea-pig, inoculated in the nostril, died on the fifth day with a cheesy deposit in the nose and septicaemia. Another guinea-pig, inoculated in the same way, but after the organism had been subcultivated repeatedly, died on the twelfth day, with caseous tracheal glands containing *B. whitmori*. A rabbit, inoculated subcutaneously, died in twenty-six hours from septicaemia with tubercles in the peritoneum. *B. whitmori* was cultivated from the peritoneum and from the heart's blood. A monkey fed on a culture remained healthy. A pony inoculated intravenously recovered after a week's fever.

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#### DESCRIPTION OF PLATES III—V.

##### PLATE III.

Fig. 1. *Barillus whitmori*. Mucoid and corrugated colonies on glycerine agar plate.

##### PLATE IV.

Figs. 2-5. Illustrating lesions in *Case 1*.

- Fig. 2. *Lung*, showing areas of consolidation consisting of minute tubercle-like nodules.  
 Fig. 3. *Liver*, showing minute suppurating tubercles and thickened gall-bladder.  
 Fig. 4. *Spleen*, the white areas are abscesses filled with creamy pus.  
 Fig. 5. *Ileum and ascending colon*, with erosions in caecum.

##### PLATE V.

Fig. 6. *Case 2*. Portion of an abscess from the liver showing the thick, necrotic wall.

(*MS. received for publication in August 1924.*—Ed.)

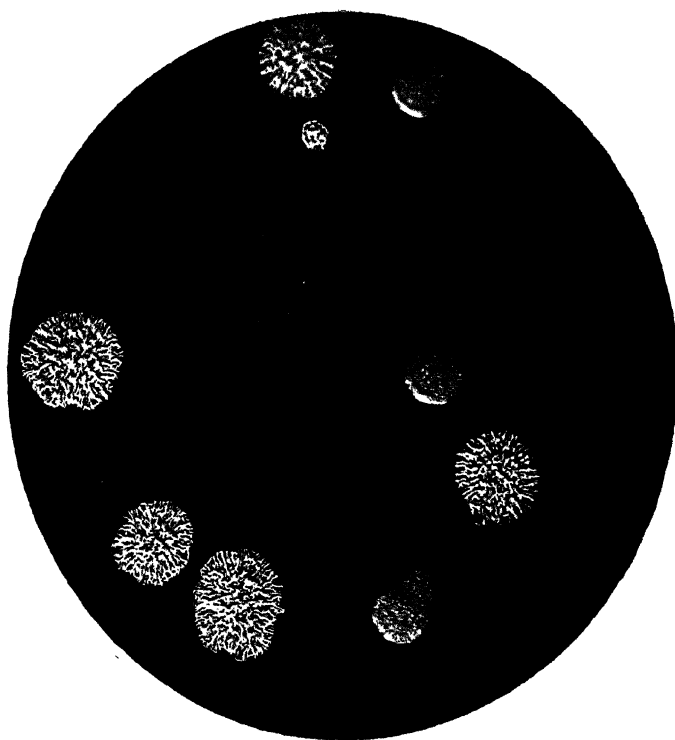


Fig. 1

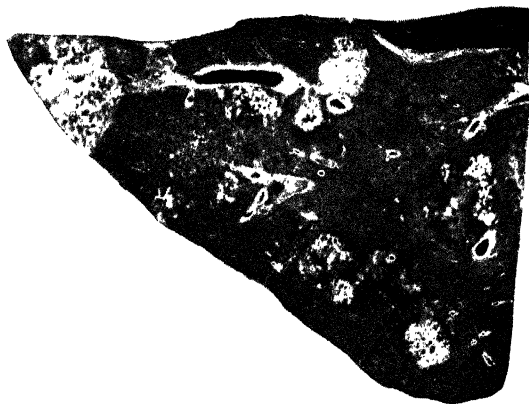


Fig. 2



Fig. 3



Fig. 4



Fig. 5







Fig. 6



## CERTIFIED MILK IN RELATION TO THE BACTERIOLOGICAL STANDARD.

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THIS paper contains an analysis of the results of the bacteriological examinations of 184 samples of milk produced by Mr W. G. Symes, Manor Farm, Fordington, Dorset, between April 1st, 1921 and June 6th, 1924.

This farmer holds a licence to produce Certified Milk which is despatched from the farm in sealed glass bottles. The samples were taken at random from the milk as it was bottled and were at once despatched by post to the laboratory, without placing in cold store. The age of the samples varied from 20-24 hours at the time of examination.

Up to August 1922 the milk was cooled with water from a town supply. From April 1921 to August 1922 the average temperature of cooling during the months May to September inclusive was 51° F. and from October to April inclusive was 48° F.

After this date the temperature of cooling seldom exceeded 50° F. and was usually between 40° F. and 50° F. Milking was done in the cowshed which is of the type ordinarily found on farms where clean milking is practised. There is no separate milking shed.

In Table I the samples have been classified according to the temperatures on arrival and the bacterial count. The presence or absence of *B. coli* is considered separately.

It is interesting to note that out of a total of 184 samples 133 or 72 per cent. showed not more than 1000 colonies per 1 c.c. even though the temperature on arrival varied from 40° F. to over 80° F., that 175 or 95 per cent. showed not more than 10,000 colonies per 1 c.c. and that 179 or 97 per cent. conformed to the certified standard of 30,000 per 1 c.c.

Of the 84 samples which arrived at temperatures between 41° F. and 55° F., 66 or 78.5 per cent. showed not more than 1000 colonies per 1 c.c. and 83 or 99 per cent. conformed to the certified standard of 30,000 per 1 c.c.

Forty-three samples arrived at temperatures varying from 56° F.-60° F.; forty-two of these showed not more than 1000 colonies per 1 c.c. and all conformed to the certified standard.

Out of a total of 137 samples which arrived at temperatures not exceeding 60° F., 108 or 78.8 per cent. showed not more than 1000 colonies per 1 c.c. and 136 or 99 per cent. showed not more than 30,000 colonies per 1 c.c.

Twenty-eight samples arrived at temperatures which lay between 61° F.

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and 65° F. Sixteen of these showed not more than 1000 colonies per 1 c.c. and 27 not more than 30,000. Of the 13 samples which arrived at temperatures varying from 66° F.-70° F. eight showed counts of 1000 or less and all were within the certified standard.

Four samples were tested at temperatures varying from 71° F.-75° F. One showed 1000 colonies per 1 c.c. and two were within the certified standard. Of the two samples which arrived at temperatures of over 75° F. one conformed to the certified standard and one showed a count which exceeded 200,000 colonies per 1 c.c. If those samples which arrived at temperatures varying from 61° F. to 75° F. or more are considered, it is seen that of the

Table I.

Temperature of samples on arrival at the laboratory	Total number of samples examined	Number of samples containing not more than					Number of samples containing more than 200,000 colonies per 1 c.c.
		1,000 colonies per 1 c.c.	10,000 colonies per 1 c.c.	30,000 colonies per 1 c.c.	50,000 colonies per 1 c.c.	200,000 colonies per 1 c.c.	
41-50° F.	32	22	32	32	32	32	0
51-55	52	44	51	51	51	52	0
56-60	53	42	53	53	53	53	0
61-65	28	16	26	27	27	27	1
66-70	13	8	12	13	13	13	0
71-75	4	1	1	2	2	2	2
Over 75	2	0	0	1*	1	1	1†
Grand total	184	133	175	179	179	180	4
		* (80° F.)		† (86° F.)			

47 tested, 25 or 53 per cent. gave counts of 1000 or less as compared with 78.8 per cent. when the temperatures on arrival lay between 40° F. and 60° F.

Further, under these conditions 43 or 91.5 per cent. showed not more than 30,000 colonies per 1 c.c. as compared with 99 per cent. when the temperatures did not exceed 60° F.

### *Presence or absence of B. coli.*<sup>1</sup>

None of the 84 samples examined at temperatures not exceeding 55° F. contained *B. coli* in 1 c.c. or less.

Of the 53 samples which were tested at temperatures varying from 56° F. to 60° F. one contained *B. coli* in 1 c.c. only, and one contained *B. coli* in 1/10 c.c. This organism was also found in 1 c.c. on two occasions out of 28 when the temperatures on testing lay between 61° F. and 65° F.

Of 13 samples examined at temperatures varying from 66° F. to 70° F. none contained *B. coli* in 1 c.c. or less.

Two out of the four samples examined at temperatures between 71° F. and 75° F. showed *B. coli* in 1/10 c.c. only; and one out of the two samples examined at temperatures exceeding 75° F. (80° F. and 86° F.) contained *B. coli* in 1/1000 c.c.

Of a total of 184 samples, 177 or 96 per cent. showed no *B. coli* in 1 c.c.

<sup>1</sup> Measured by the standard adopted by the Ministry of Health.

and 7 or 3·7 per cent. showed *B. coli* in 1 c.c. or less, 3 or 1·6 per cent. showed *B. coli* in 1 c.c. but in no less quantity, 3 or 1·6 per cent. showed *B. coli* in 1/10 c.c. but in no less quantity, and 1 or 0·5 per cent. showed *B. coli* in 1/1000 c.c.

A consideration of the temperatures of the samples on arrival shows that under ordinary conditions of transport some form of artificial cooling is necessary since four (8·7 per cent.) of the 47 samples which reached the laboratory at temperatures exceeding 60° F. failed to maintain the certified standard, whereas there was only one failure (0·7 per cent.) among 137 samples which arrived at temperatures not exceeding 60° F.

The results summarised above are so good that the conclusion that the failures to conform to the certified standard were due solely to adverse temperature effects, appears to be justified.

The figures used in a previous paper<sup>1</sup> which showed the results of the examinations of 82 samples of milk taken between November 1916 and September 1918 may be combined with those analysed in this present paper to extend the number of examinations of certified milk to a period of more than five years. With this addition the total number of samples examined at approximately 24 hours after milking amounts to 266 (184 + 82).

Of the series of 82 samples two or 2·44 per cent. failed to maintain the certified standard and of the present series of 184 samples 5 or 2·7 per cent. gave counts in excess of 30,000 per 1 c.c.

When these two series are combined it is seen that of a total of 266 samples 7 or 2·6 per cent. gave counts in excess of 30,000 per 1 c.c.

When the incidence of *B. coli* is considered it is found that of the first series of 82 samples 7 or 8·5 per cent. showed acid and gas in 1 c.c. or less and that of the series of 184 samples 7 or 3·7 per cent. showed the presence of *B. coli* in 1 c.c. or less. Therefore out of a total of 266 samples 14 or 5·2 per cent. were found to contain *B. coli* in 1 c.c.

This does not mean that on this basis 5·2 per cent. failed to maintain the certified standard of no acid and gas in 1/10 c.c. since seven of the samples showed *B. coli* in 1 c.c. but in no less quantity.

The results of the examinations of Mr Symes' milk serve to emphasise the fact that if delivery can be made within 24 hours there is little danger that the certified standard will not be maintained, provided that the temperature does not exceed 60° F.

Further it is shown that success in clean milk production rests, not so much on the buildings and equipment as upon the skill of the workers and the unflagging interest of the farmer.

<sup>1</sup> Freear, Mattick and Stenhouse Williams (1921). "A Study of the Bacteriological Examination of Grade 'A' (Cert.) Milk." *Journ. of Hyg.* xx. 125.

# MALARIA IN RURAL SETTLEMENTS IN PALESTINE.

## 1. INCIDENCE AND ETIOLOGY OF MALARIA.

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(With Plates VI-IX, 5 Charts and 1 Map.)

### INTRODUCTION.

THE impression has always prevailed, that malaria is the most common disease in Palestine, and that it is more or less highly prevalent in all of rural Palestine. This impression, based as it is on mass experience, is in general correct; but it lacks the certainty derived from statistical data.

In 1921 Kligler and Weitzman<sup>(3)</sup> made a study of the malaria conditions in two selected areas in lower Galilee and conducted control demonstrations in those areas with very satisfactory results. In 1922 the work was extended to include two areas in Judea. In 1923 a more comprehensive comparative study was undertaken of the malaria prevalence in various sections of the country and control demonstrations were organised in nine typical districts located in different parts of Palestine.

The principal aim of this work was to ascertain (1) the actual prevalence and cause of malaria in different sections of rural Palestine; (2) whether it was possible to bring the disease under control at a reasonable cost.

The purpose of this paper is to summarise the results of these studies. We shall attempt to present as briefly as possible the information obtained regarding (1) the incidence and etiology of malaria in rural Palestine; (2) the value of blood and spleen examinations as indices of malaria prevalence; (3) the nature of the swamps and the types of mosquitoes responsible for the high malaria incidence; and (4) the method of organisation of the demonstration areas, the methods of control employed, the effects of the control measures and the cost of control.

*Incidence of Malaria.* In a highly malarious country like Palestine, where the public is more or less habituated to the disease and uses quinine freely for any form of fever, it is exceedingly difficult to obtain reliable statistics of malaria incidence. This difficulty is even greater in the rural than in the urban communities. Consequently there are few reliable figures available indicating the actual incidence of the disease.

One of our tasks when the work was started was to organise regular reporting of malaria cases. At the commencement of the work in a given section, we obtained the doctor's records, the laboratory findings, if any were

<sup>1</sup> Aided by a grant from the American Joint Distribution Committee, New York City.

Chart 1. Comparison of Percentage Malaria incidence in the Demonstration Areas in 1922 and 1923.

השוואה של אחוזים מקרי המלריה במחוזי הקונטרול בשנות 1922-1928

New  
Total

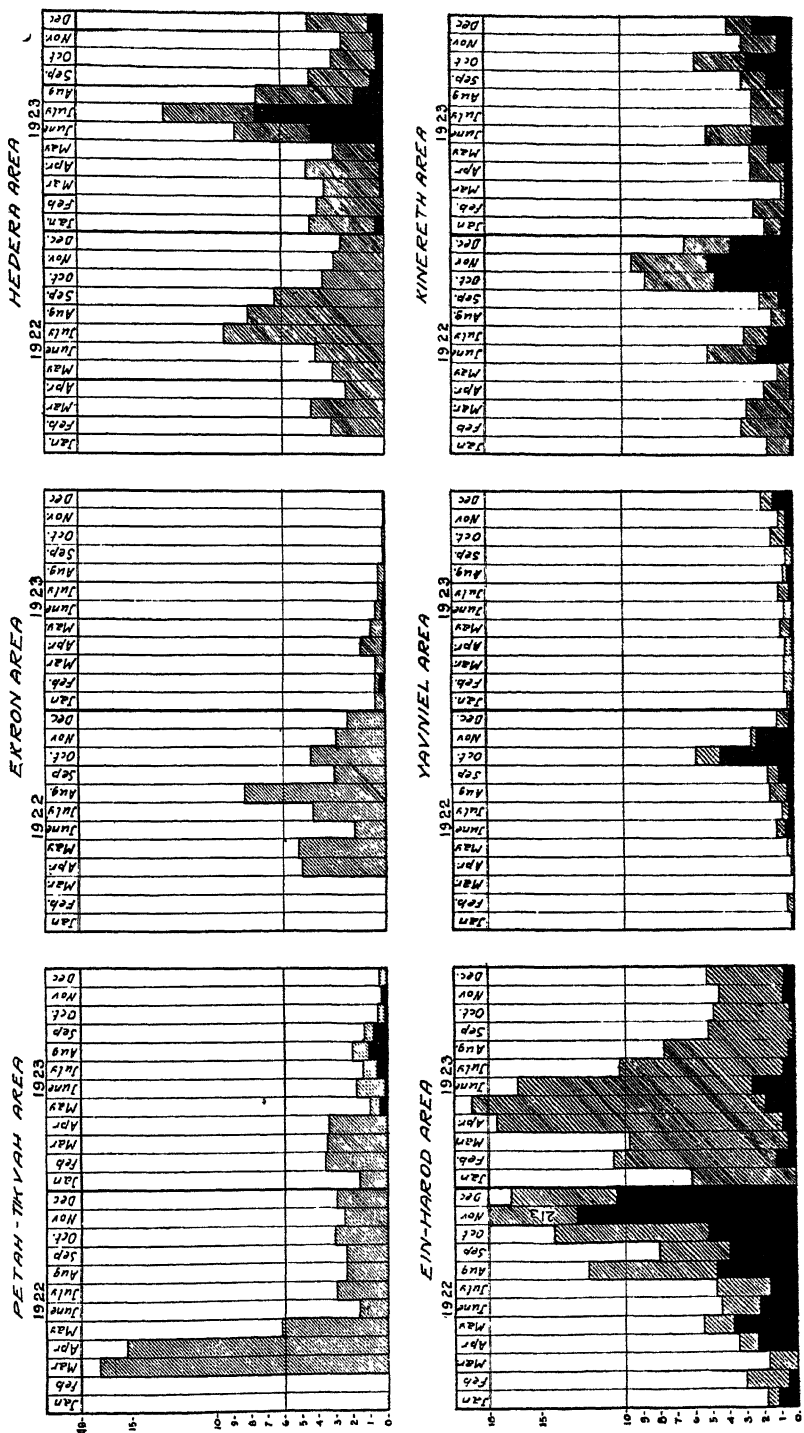
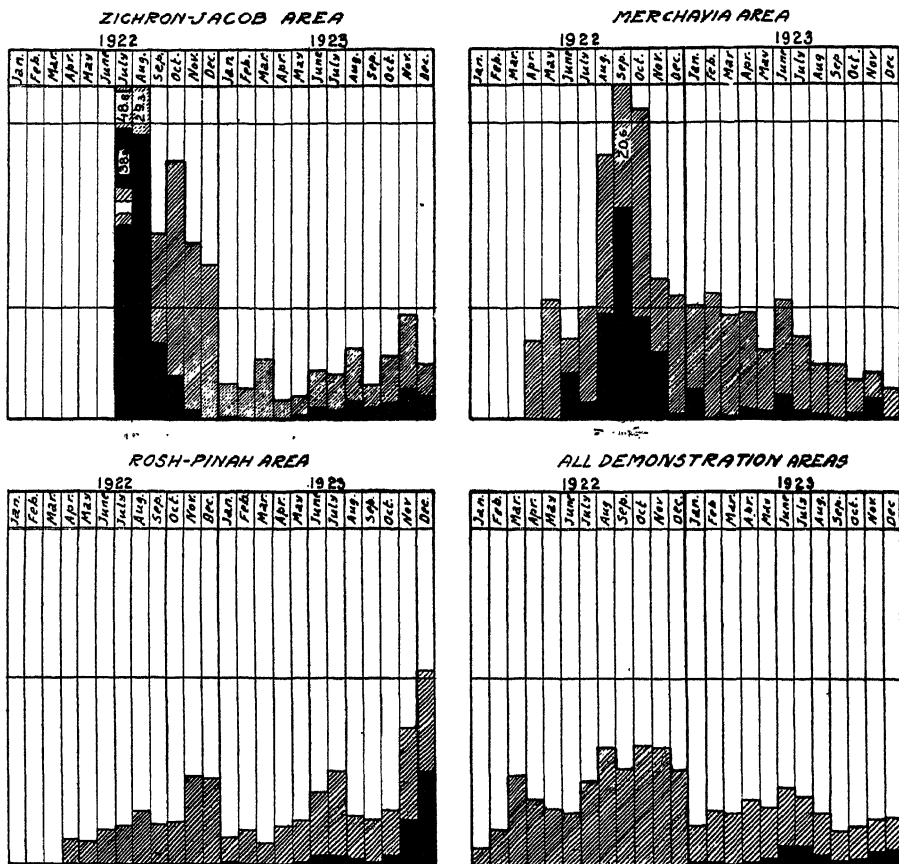




Chart 1—continued



Note. In 1922 the returns were not always differentiated into primary and secondary cases. Where such information was obtained it is indicated in the chart; where not, only the total incidence is given. For 1922 the incidence is given only for those places from which reliable records were obtained.

available and the personal history of each inhabitant. Malaria cards were prepared for each individual. In this manner we obtained a composite record of the malaria cases during a greater or lesser period prior to the commencement of our work. Thereafter the malaria inspector paid regular weekly visits to the doctors or nurses of each village or settlement and obtained from them the names of all the malaria cases. These individuals were interviewed and their histories obtained. Our work was facilitated by the fact that nearly every Jewish settlement or group of smaller settlements, consisting of over 100 population, had a resident doctor or nurse.

Thus it was possible during 1922 and more effectively in 1923 to gather fairly accurate data of the malaria incidence in the Jewish villages included in our demonstration areas. Similar data could not unfortunately, for obvious reasons, be obtained from the Arab villages within those areas; but since the Jewish population formed about 65 per cent. of the total, and the incidence tables for 1923 are based on a total population of over 10,000, these figures may be considered as representative. The records for 1923 are naturally more complete and more accurate than are those for 1922. But despite the lack of completeness in certain details, the 1922 figures give a good picture of the prevalence of malaria in the various demonstration areas before active control was organised and serve as a satisfactory basis for estimating the degree of effectiveness of the control measures carried out during 1923.

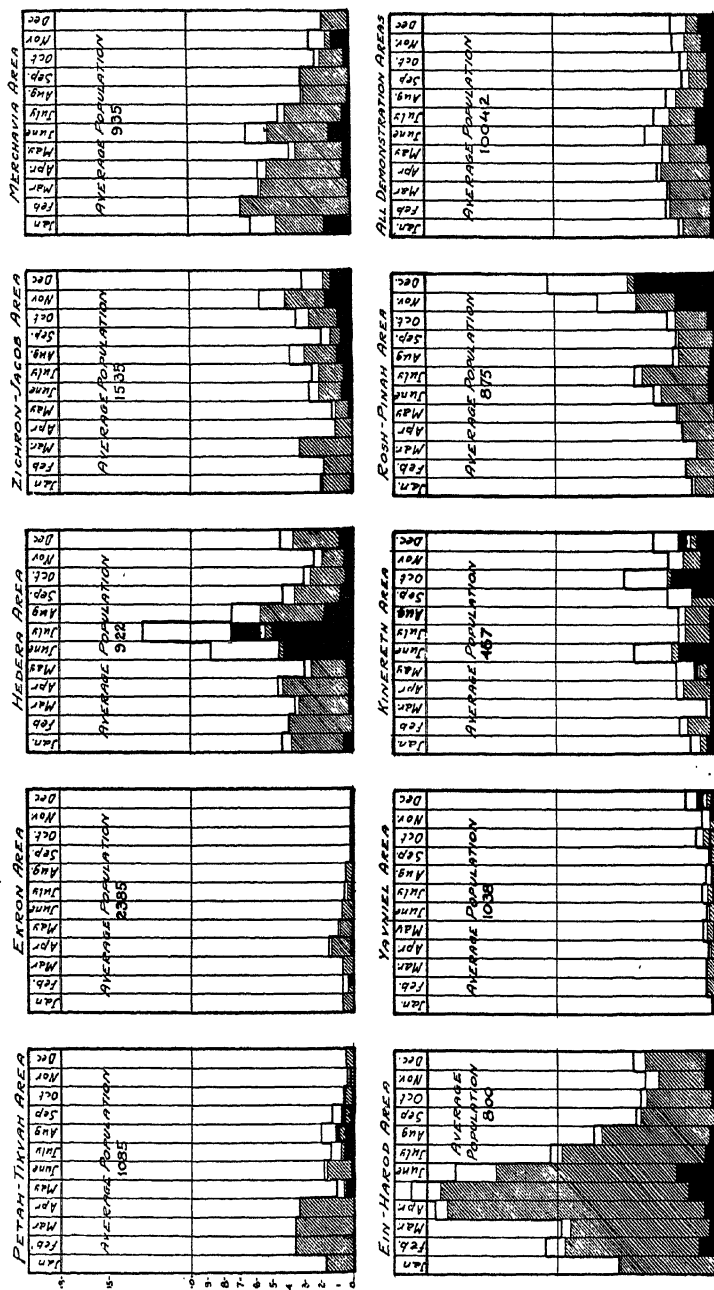
The total monthly percentage incidence of all forms of malaria in the various demonstration areas are shown in a series of tables and charts. In Chart 1 is presented the total monthly percentage incidence of malaria in 1922 and 1923. Wherever the data was sufficiently reliable the incidence of primary cases is also given.

In Tables I and II and their corresponding charts (Charts 2 and 3) are presented more detailed analysis of the incidence data for 1923. In the former are given the incidence of primary and secondary cases and in the latter the relative frequency of occurrence of the various types of malaria parasites. It is not an easy matter to differentiate between primary and secondary cases of malaria in people, who have already had an attack before. We usually based our judgment on certain definite criteria. During the winter and early spring primary cases may be practically, though not completely, excluded; during this period relapses of benign tertian malaria commonly occur among those who have had malaria during the previous summer and fall. All such cases may, therefore, be safely classed as relapses. All cases of malignant tertian malaria in the summer and fall months were considered new infections, if it was the first attack of that form of malaria during that season. Benign tertian cases were considered new infections if the patient had had no attack of malaria for a year prior to the present illness. We believe that this basis of differentiation corresponds fairly closely with the facts and yields a reasonably accurate classification of cases.

The differentiation of the various types of parasites was made, whenever

Chart 2. Monthly percentage incidence of primary and secondary cases of Malaria in the Demonstration Areas, Year 1923.

### אחוזים חדשים של מקרי מלריה חדשים וחוזרים במחוזי הקונטרול בשנת 1923



Legend.

Primary cases

Secondary cases

Total

באור :

מקרים חדשים

חוזרים

סך

Note. Average population = mean monthly population for the entire year = sum of the population of each month divided by the total number of months.

Table I.

Area Form	Pet Tikvah			Ekron			Hadera			Z. Jacob			Mercharia			E. Harod			Yavniel			Kinereth			R. Pinah			Alldem Ar.		
	New	Relapse	Total	New	Relapse	Total	New	Relapse	Total	New	Relapse	Total	New	Relapse	Total	New	Relapse	Total	New	Relapse	Total	New	Relapse	Total	New	Relapse	Total			
Jan.	0.0	1.7	1.7	0.0	0.6	0.6	0.6	3.8	4.3	0.1	1.6	1.9	1.6	4.6	6.2	0.0	6.1	6.1	0.2	0.1	0.3	0.6	1.0	1.6	0.1	1.3	1.4	0.2	1.9	2.1
Feb.	0.0	3.6	3.6	0.3	0.3	0.6	0.0	3.9	3.9	0.0	1.7	1.7	0.0	6.8	6.8	1.2	9.6	10.8	0.0	0.6	0.6	0.4	1.8	2.2	0.0	1.8	1.8	0.2	2.7	2.9
Mar.	0.0	3.3	3.5	0.0	0.6	0.6	0.2	3.3	3.5	0.0	3.3	3.2	0.1	5.5	5.6	0.5	9.2	9.7	0.0	0.6	0.6	0.4	0.2	0.6	0.0	1.1	1.1	0.1	2.7	2.8
Apr.	0.0	3.4	3.4	0.1	1.3	1.5	0.2	4.3	4.3	0.0	1.0	1.0	0.5	5.2	5.7	0.8	16.8	17.6	0.1	0.4	0.5	0.4	2.0	2.4	0.0	2.0	2.0	0.2	3.3	3.5
May	0.4	0.6	1.0	0.1	0.8	0.9	0.4	2.5	2.9	0.2	1.0	1.2	0.4	3.3	3.7	1.8	17.2	19.0	0.2	0.6	0.8	1.4	1.0	2.4	0.1	2.2	2.3	0.4	2.7	3.1
June	0.1	1.6	1.7	0.2	0.5	0.7	4.2	4.4	8.6	0.6	2.0	2.6	1.3	5.1	6.4	2.6	13.8	16.4	0.1	0.5	0.6	2.3	2.7	5.0	0.5	3.4	3.9	1.1	3.1	4.2
July	0.6	0.8	1.4	0.1	0.4	0.5	7.5	5.4	12.9	0.5	2.0	2.4	0.4	4.0	4.4	0.8	9.7	10.5	0.3	0.6	0.9	0.4	1.9	2.3	0.5	4.5	5.0	1.1	2.7	3.8
Aug.	1.1	0.9	2.0	0.0	0.4	0.4	1.7	5.8	7.5	0.9	2.9	3.8	0.1	2.8	2.9	0.5	7.2	7.7	0.4	0.3	0.7	0.4	1.9	2.3	0.4	2.2	2.6	0.6	2.3	2.9
Sept.	0.3	0.5	1.3	0.0	0.1	0.1	0.7	3.6	4.3	0.6	1.3	1.9	0.0	2.9	2.9	0.2	4.8	5.0	0.1	0.4	0.5	1.5	1.5	3.0	0.1	2.2	2.3	0.3	1.5	1.8
Oct.	0.1	0.5	0.6	0.0	0.1	0.1	0.4	2.6	3.0	0.8	2.6	3.4	0.3	1.8	2.1	0.4	4.4	4.8	0.5	0.8	1.3	2.7	3.0	0.5	2.4	2.9	0.4	1.6	2.0	
Nov.	0.2	0.1	0.3	0.0	0.1	0.1	0.5	1.9	2.4	1.6	4.1	5.7	1.1	1.4	2.5	0.8	3.7	4.5	0.5	0.5	1.0	0.9	2.0	2.9	2.4	4.9	7.3	0.8	1.8	2.6
Dec.	0.0	0.4	0.4	0.0	0.1	0.1	0.8	3.7	4.5	1.2	1.7	2.9	0.0	1.6	1.6	0.7	4.5	5.2	1.2	0.6	1.8	2.3	1.6	3.9	3.0	5.4	10.4	0.9	1.7	2.6

Chart 3. Monthly percentage incidence of the three types of Malaria in the Demonstration Areas. Year 1923.

### אחוזים חדשים של שלשת מיני המלריה במחוזי הקונסולרלה בשנת 1923

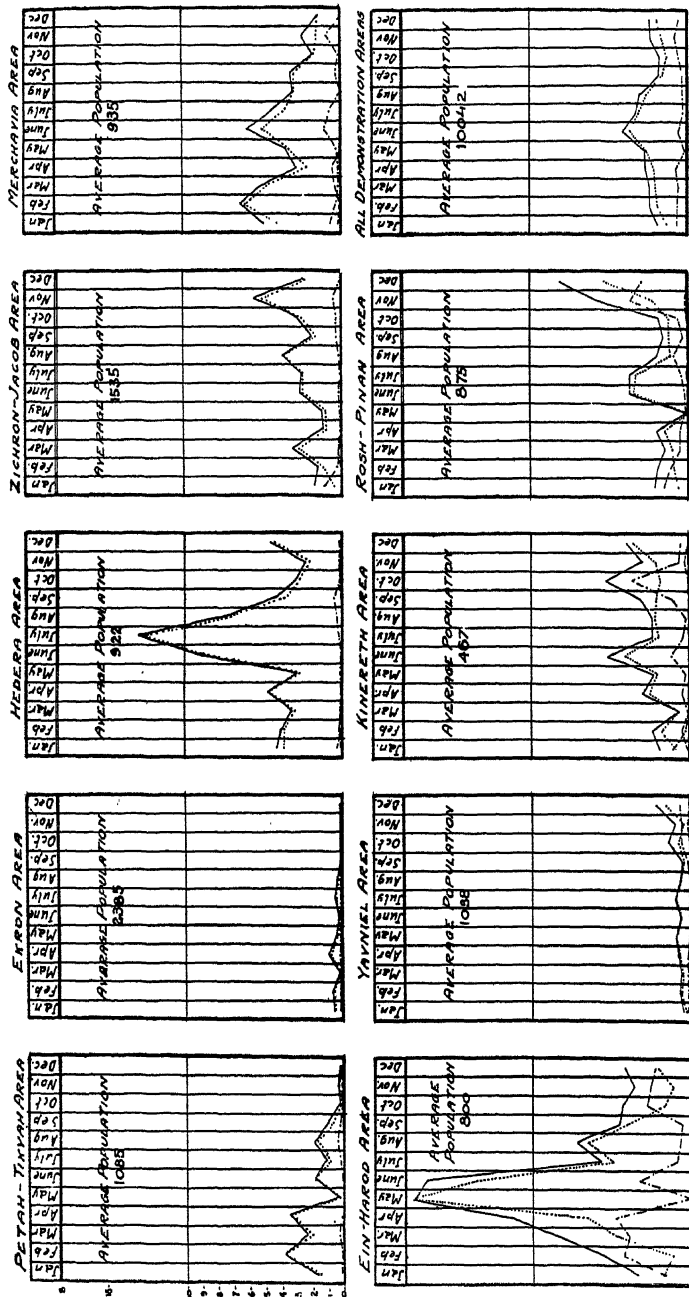


Table II.

Area	Pet Tikvah			Ekron			Hedera			Z. Jacob			Merchavim			E. Harod			Yavniel			Kinereth			R. Pinah			All Demar.										
Type	Tertian	Quartan	Total	Tertian	Quartan	Total	Tertian	Quartan	Total	Tertian	Quartan	Total	Tertian	Quartan	Total	Tertian	Quartan	Total	Tertian	Quartan	Total	Tertian	Quartan	Total	Tertian	Quartan	Total	Tertian	Quartan	Total								
Jan.	1.50	1.00	1.7	0.50	1.00	0.6	3.80	0.4	0.4	2.2	0.5	1.1	0.1	1.7	4.0	0.7	0.1	4.8	1.7	1.6	0.3	3.3	0.2	0.1	0.0	0.3	0.8	1.0	0.0	1.8	0.6	0.0	1.8					
Feb.	3.60	0.00	3.6	0.50	0.01	0.6	3.80	0.0	1.3	9.1	1.1	0.4	0.0	1.5	6.0	0.3	0.6	6.3	2.2	4.2	0.0	5.4	0.5	0.1	0.0	0.6	1.6	0.2	0.4	2.2	0.8	1.0	0.1	1.8				
Mar.	2.00	3.00	2.3	0.1	0.00	0.1	3.10	0.0	1.3	2.7	0.2	0.2	3.1	4.70	1.0	3.5	1.4	5.3	9.0	3.8	4.0	0.1	0.0	0.5	0.6	0.0	0.6	0.9	0.5	0.0	1.4	1.9	0.5	0.0	2.4			
Apr.	3.40	0.00	3.4	0.70	2.00	0.9	4.60	0.0	4.6	1.0	0.1	0.1	1.2	0.1	0.5	2.7	6.5	4.7	0.0	11.2	0.6	0.1	0.0	0.7	2.4	0.4	0.0	2.8	1.4	0.5	0.0	1.9	2.0	0.6	0.0	2.6		
May	0.30	0.00	0.3	0.30	1.00	0.4	2.70	1.0	0.2	8.1	0.2	0.0	1.3	3.2	0.2	0.3	3.4	17.6	0.0	0.0	17.6	0.8	0.0	0.0	0.8	2.0	0.2	0.0	2.2	0.0	0.1	0.0	0.1	2.50	1.0	0.2	6	
June	1.80	0.00	1.8	0.20	1.00	0.3	3.80	0.0	0.8	2.4	0.2	0.0	2.6	4.9	1.0	0.0	5.9	3.5	3.2	0.0	6.7	0.6	0.0	0.0	0.6	4.0	1.1	0.0	5.1	3.4	0.2	0.0	3.6	3.6	0.5	0.0	4.1	
July	0.90	0.20	0.1	0.4	0.1	0.0	0.5	12.0	3.0	0.12	9.2	4.0	0.0	2.4	3.6	0.7	0.4	4.3	4.8	0.9	0.0	5.7	0.9	0.0	0.0	0.9	1.9	0.4	0.0	2.3	3.2	0.4	0.0	3.6	2.9	0.3	0.0	3.2
Aug.	1.50	0.40	0.1	0.3	0.1	0.0	0.4	7.0	4.0	0.07	3.7	0.1	0.0	3.8	2.9	0.0	0.0	2.9	6.50	0.0	7.1	0.6	0.0	0.0	0.6	2.1	0.2	0.0	2.3	1.1	0.8	0.0	1.9	2.5	0.3	0.0	2.8	
Sept.	0.70	0.30	0.1	0.1	0.00	0.1	3.70	0.6	0.4	3.3	1.6	0.3	0.0	1.9	2.8	0.3	0.0	3.1	4.0	5.00	4.5	0.4	0.1	0.0	0.5	2.0	1.0	0.0	3.0	1.1	0.3	0.0	1.4	1.5	0.3	0.0	1.8	
Oct.	0.20	1.00	0.3	0.1	0.00	0.1	2.70	2.0	0.1	3.0	2.6	0.3	0.0	2.9	1.7	0.1	0.1	1.8	16.2	7.00	4.3	0.7	0.5	0.0	1.2	1.7	3.5	0.0	5.2	1.1	0.6	0.0	1.7	1.2	0.5	0.0	1.7	
Nov.	0.30	1.00	0.4	0.1	0.00	0.1	2.10	2.0	0.1	2.4	5.0	0.5	0.0	5.5	1.5	0.9	0.0	2.4	1.0	2.5	0.0	3.5	0.4	0.4	0.1	0.9	2.0	0.7	0.2	2.9	2.1	3.5	0.1	5.7	1.5	0.7	0.0	2.2
Dec.	0.20	1.00	0.3	0.1	0.00	0.1	4.30	0.0	2.4	5.2	3.0	1.0	2.4	1.4	0.0	0.0	1.4	1.9	2.2	0.0	4.1	1.4	0.6	0.0	2.0	3.4	0.5	0.0	3.9	5.2	2.8	0.0	8.0	1.8	0.5	0.0	2.3	

possible, from the thick drop which we used in all our routine work. But usually both thick drops and smears were taken, and in case of doubt the smear was also examined. The relative frequency of occurrence of the various malaria parasites as shown in the chart summarising our laboratory findings (Chart 4) may, therefore, be considered as typical.

An examination of these tables and charts reveals a number of important facts regarding the incidence and epidemiology of malaria in rural Palestine: (1) During 1922 malaria was highly prevalent, particularly, wherever control was not being carried out. (2) In all of the districts malaria was endemic, and in most of them it was also epidemic during 1922. (3) In some of the areas (Hedera, Zichron), the epidemics occur in June and July; in others (Yavniel) they occur in October and November; while in some (Ein Harod, Kinereth) during both of these periods. (4) The benign tertian form of malaria is present throughout the year, but is relatively more abundant than the malignant type during the months of January to September than during the last months of the year; the malignant tertian or tropical form of malaria is most common in the late summer and autumn—October to December inclusive. (5) Malaria relapses occur throughout the year, but mostly during the winter and spring months—February to May inclusive; corresponding with the high prevalence of the benign tertian form of malaria.

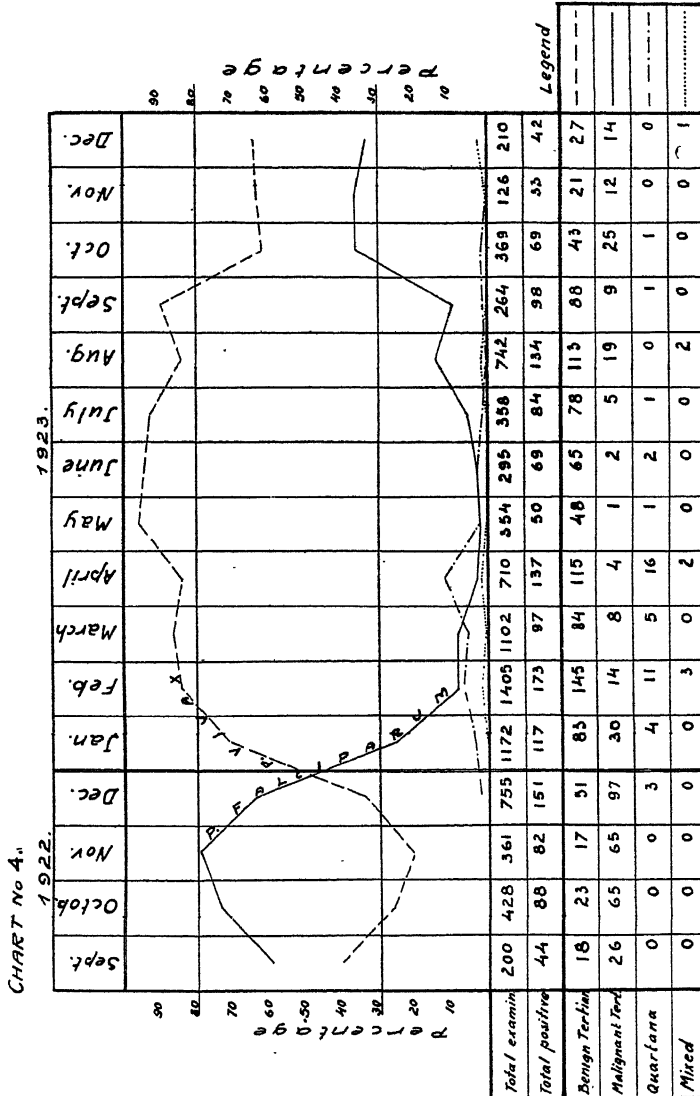
*Prevalence of Malaria.* The high prevalence of malaria in all of the Demonstration Areas prior to our control is clearly indicated in Chart 1. In nearly all, except the control districts, the average monthly incidence for 1922 was 4 per cent. or over, indicating a minimal annual malaria incidence of 50 per cent. and an incidence of over 100 per cent. in at least three of the districts. There can be little doubt, therefore, that malaria was the most prevalent disease in these districts.

*Endemic and Epidemic Malaria.* The noteworthy fact is that malaria exists in both endemic and epidemic form. Endemic malaria exists throughout the year. It represents the residue of uncured malaria, which recurs periodically, but most frequently during the winter and spring months. Epidemic malaria occurs at two distinct periods, with a short interval of quiescence between them. The first epidemic period is from the middle of June to the end of July or middle of August, and consists chiefly of the benign tertian form of malaria. The second epidemic period extends from the end of September, or early October, to about the middle of December and consists mainly of tropical or malignant tertian malaria. In Hedera, Petach Tikwa and Zichron districts, situated in the Coastal Plain, the epidemics usually occur in the summer. In the Yavniel districts the principal epidemic is in the fall. In the Valley of Esdraelon and Kinereth regions, epidemics are apt to occur both in the summer and autumn (see Charts 1 and 2).

*Seasonal Prevalence of Benign and Malignant Malaria.* A study of Table II and Chart 3 reveals the fact that there is a distinct periodicity in the prevalence of benign tertian and malignant or tropical forms of malaria. This

is brought out even more emphatically in the graph (Chart 4), showing the results of the blood examinations in our laboratory from September 1922 to December 1923. There are apparently three distinct periods. The first period, covering the months of February to June (corresponding with the period of

Chart 4. Incidence of *P. vivax* and *P. falciparum* in laboratory examinations.



relapses, with only sporadic primary cases), consists almost entirely of benign tertian malaria. The second period (corresponding with the first epidemic period) extends from the middle of June to the end of August and consists chiefly of benign tertian, with occasional cases of malignant tertian, or



tropical, form of malaria. In the third period, the last three months of the year, there is a sharp rise in the cases of malignant tertian, or tropical malaria, with a proportionate fall in the incidence of the benign tertian form of the disease. This last period corresponds with the second epidemic season. Quartan malaria was rare, but was found in considerable numbers during the months of March and April, in the course of the general blood examinations.

This seasonal incidence of the different types of malaria corresponds closely with that reported by Wenyon<sup>(5)</sup> for Macedonia. Wenyon found that *P. falciparum* constituted about half of the total positive laboratory findings in November and fall to about 2 per cent. in March. The incidence chart (Chart 3) of the control areas and particularly the one showing our laboratory findings (Chart 4) show practically the same relationship. The laboratory chart shows also a marked difference between the periods September to December 1922 and 1923, respectively; this difference is due to the greater prevalence of malignant tertian epidemics in 1922.

The explanation of this phenomenon which seems to us most tenable, is the one proposed by Wenyon<sup>(5)</sup>. A similar explanation was also advanced by us<sup>(3)</sup>. This explanation is based on the difference in the reaction of the benign and malignant tertian parasites and their respective gametes to quinine<sup>(1,2,4,5)</sup>. The greater resistance of the benign tertian chizonts to quinine seems fairly well established. This resistance accounts for the abundance of relapses during the winter and early spring months, when mosquitoes are not yet fully active, and new infections rare. With benign tertian the only existing type of malaria, it is clear why the summer epidemic consists chiefly of benign tertian malaria. With continued subjection to quinine, however, the benign tertian gametes become uninfective for the mosquito, while the malignant gametes remain unaffected. Consequently the tropical form of malaria increases, first slowly then more rapidly, reaching a peak in November, and then as rapidly declines partly because of treatment, but mainly perhaps because the mosquitoes cease their activity about the beginning or the middle of December, according to the onset of the rainy season. It is also possible that the decrease in benign tertian cases during the autumn is only apparent, due to the masking action of the tropical form of malaria. That this is more than a mere supposition is indicated by the usual appearance of large numbers of tertian relapses after an epidemic of malignant malaria.

#### SUMMARY.

Statistical and laboratory data are presented, which bear on the incidence and epidemiology of malaria in rural Palestine. These data indicate that the annual incidence of malaria ranged between 50 and 100 per cent., and that in the areas where no control was effected in 1922 it was always considerably higher than the minimum figure. They also show that malaria is both endemic and epidemic and that there are two epidemic periods, one in the summer and one in the fall of the year. In the winter and spring there is little or no

primary malaria. The parasites most frequently encountered are *P. vivax* and *P. falciparum*; *P. malariae* is uncommon. The *P. vivax* is found throughout the year, but constitutes about 98 per cent. of the total malarias in February to June and only 50–60 per cent. during October to December.

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## 2. THE PARASITE AND THE SPLEEN RATES.

BY I. J. KLIGLER, J. M. SHAPIRO AND I. WEITZMAN.

It is fairly generally acceded that parasite and spleen<sup>(1,2,3)</sup> rates are reliable indices of the prevalence of malaria in a given community. Consequently, at the commencement of our control work in 1922 and 1923, we made a general blood and spleen examination of groups of inhabitants in each of the Demonstration Areas. The object of these examinations was: first, to obtain supplementary evidence as to the prevalence and distribution of malaria in the Control Zones and second, to detect and treat as many of the carriers as possible, as a first step in our control work.

It was impossible, for various reasons, to examine all of the 20,000 inhabitants in the control areas. The size of the task was too large and, moreover, the native population does not readily submit to examination either of blood or spleen; women as a class have to be excluded, almost entirely, and men are often away for days or weeks in the fields. We examined over 5000 blood preparations and over 3000 spleens. From the Jewish population we examined representative groups of men, women and children. Among the Moslem population we examined most of the children and as many men as could be mustered together for the show. Blood and spleen examinations in a Moslem village in Palestine is a special event which begins with sitting about in the Sheik's house, talking, drinking coffee and talking some more. Only after these preliminaries are over and after the first man, brave enough to have himself stabbed for blood, has been found, does the work begin in earnest. The first "victim" is always sure to bring others.

All of the examinations were made during the months of February, March and April. These months were selected intentionally, because they are the

months when recurrences of malaria are most common, and when new infections can be practically excluded. Examinations made during these months furnish, therefore, a better picture of the actual extent of the latent malaria in a community.

For the blood examinations we used the thick drop method, stained with Giemsa. Usually only drops were taken; but frequently both drops and smears were taken, but the smears were examined only when the differentiation of the parasite was doubtful. Many of the examinations were made in the field, but more often the slides were brought to the laboratory.

The spleens were examined with the patient reclining, and the knees flexed. The enlargements were designated as palpable, one, two, etc., according to the number of finger breadths from the border of the spleen to the costal margin. All spleens that were definitely palpable were classed as enlarged.

The results of these examinations are summarised in Tables III and IV. The first of these tables contains the total blood and spleen incidence of Jews and Arabs in each demonstration area. The second table gives a more detailed comparison of the blood and spleen rates among the Jews and Arabs in the same locality. In Table III the blood rates are given for the population as a whole, but in Table IV they are analysed for age groups; the spleen rates are given separately for adults and for children below fourteen.

It is difficult to analyse these figures and arrive at any definite conclusions. Both the high blood and spleen rates, independently and together, point to a high malaria incidence. On superficial analysis they also suggest that the malaria incidence is higher among the Arab than among the Jewish population. A closer examination, however, of the figures obtained in different settlements in the same locality throws doubt on this interpretation. There is no justification for assuming that two groups of people living at equidistance from the same swamp would be differently affected. But this would be the case with the villages listed in Table IV, if we were to accept the blood and spleen rates as indices of the relative prevalence of malaria.

It is apparent, therefore, that in Palestine, at least, there are factors other than malaria incidence, which modify the validity of either the blood or the spleen rate as an index of malaria prevalence. The parasite rate, for example, may be greatly affected by the quinine consumption, either as a prophylactic, or for treatment. In a number of settlements, which had a high malaria incidence in 1922 but received good, intensive, treatment we found a low parasite rate. Conversely, other places, which had a relatively lower malaria incidence in 1922 and received poor treatment gave a higher parasite rate. The same criticism applies to the spleen rates. The spleen rate is affected not only by the incidence of malaria and the effectiveness of treatment, but also by the length of time exposed to infection.

As a result of our findings we believe that either blood or spleen rates are of value as general indices of malaria prevalence in the Arab communities, but this is not the case in the Jewish villages. The peasant or fellah uses very

little quinine prophylactically, and the treatment he receives is largely sporadic and symptomatic. He rarely receives thorough, systematic, treatment. Consequently the children have a high parasite rate and a high or low spleen rate, according to the length of time exposed to the infection; while the older people

Table III.

*Comparison of Blood and Spleen Rates in Various Districts.  
February-March, 1923.*

<i>Blood Index.</i>										
District		Number examined			Number positive			Percentage positive		
		Jews	Arabs	Total	Jews	Arabs	Total	Jews	Arabs	Total
Ecron		679	296	975	39	54	93	5.7	18.2	9.5
Petach Tikwa.		628	42	670	36	8	44	5.7	19.0	6.6
Hedera		441	0	441	24	0	24	5.4	—	5.4
Zichron		177	0	177	32	0	32	18.1	—	18.1
Merchavia.		762	221	983	48	57	105	6.3	25.8	10.7
Nuris		338	0	338	30	0	30	8.9	—	8.9
Yavniel		410	146	556	9	2	11	2.2	1.4	2.0
Kinereth		403	0	403	17	0	17	4.2	—	4.2
Rosh Pina		631	100	731	16	16	32	2.5	16.0	4.4
<i>Spleen Index.</i>										
Ecron	Adults	327	66	393	65	46	111	19.9	69.7	28.2
	Children	157	217	374	36	179	215	22.9	82.5	57.5
	Total	484	283	767	101	225	326	20.9	79.5	42.5
Petach Tikwa.	Adults	445	14	459	148	14	162	33.3	100.0	35.3
	Children	135	28	163	57	27	84	42.2	96.4	51.5
	Total	580	42	622	205	41	246	35.3	97.6	39.6
Hedera	Adults	237	0	237	123	0	123	51.9	—	51.9
	Children	96	0	96	14	0	14	14.6	—	14.6
	Total	333	0	333	137	0	137	41.1	—	41.1
Zichron	Adults	112	0	112	47	0	47	42.0	—	42.0
Merchavia.	Adults	346	58	404	72	10	82	20.8	17.2	20.3
	Children	79	58	137	15	30	45	19.0	51.7	32.9
	Total	425	116	541	87	40	127	20.5	34.5	23.5
Nuris	Adults	88	0	88	22	0	22	25.0	—	25.0
Yavniel	Adults	173	0	173	22	0	22	12.7	—	12.7
	Children	121	0	121	29	0	29	24.0	—	24.0
	Total	294	0	294	51	0	51	17.4	—	17.4
Kinereth	Adults	254	0	254	78	0	78	30.7	—	30.7
	Children	62	0	62	13	0	13	21.0	—	21.0
	Total	316	0	316	91	0	91	28.8	—	28.8
Rosh Pina	Adults	205	23	228	58	15	73	28.3	65.2	32.0
	Children	83	51	134	28	35	63	33.7	68.6	47.0
	Total	288	74	362	86	50	136	29.9	67.6	37.6

who slowly develop a resistance to the parasite have a lower parasite rate than the children, and sometimes even a lower spleen rate, due to the hard shrivelled spleen which is not palpable. The Jewish farmers, on the other hand, use quinine very freely, seek medical aid frequently, especially for their children, and as a rule obtain fairly good treatment. The effect is registered in relatively lower blood and spleen rates. In both groups of the population, blood

and spleen rates misrepresent the actual condition; in the one they exaggerate, in the other they minimise the actual degree of prevalence of the disease.

The analysis of our figures with a knowledge of the malaria conditions as obtained from the incidence records leads us to believe that, while the blood and spleen when used independently are not satisfactory indicators, they may be of considerable value when they are correlated with respect to the age groups in the community.

Table IV.

*Comparison of Blood and Spleen Indices among Jews and Arabs of the same locality. February-March, 1923.*

		Number examined			Number positive			Percentage positive		
Place		Adults	Children	Total	Adults	Children	Total	Adults	Children	Total
Ecron	Jews	215	110	325	17	11	28	7.9	10.0	8.6
	Arabs	52	139	191	6	42	48	11.5	30.3	25.1
Gedera	Jews	77	43	120	3	4	7	3.9	9.3	5.9
	Arabs	2	46	48	0	5	5	0.0	10.9	10.4
Ein Ganim	Jews	255	118	373	10	6	16	3.9	5.1	4.3
	Arabs	14	28	42	1	7	8	7.1	25.0	19.0
Merchavia	Jews	162	0	162	16	0	16	9.9	—	9.9
	Arabs	185	0	185	54	0	54	29.2	—	29.2
Yessod	Jews	155	83	238	2	9	11	1.3	10.8	4.7
	Arabs	36	38	74	3	9	12	8.4	23.7	16.2
Ayeleth	Jews	50	0	50	1	0	1	2.0	—	2.0
	Arabs	13	13	26	1	3	4	7.7	23.0	15.4

		Number examined			Number positive			Percentage positive		
Place		Adults	Children	Total	Adults	Children	Total	Adults	Children	Total
Ecron	Jews	160	85	245	14	8	22	8.7	9.4	9.0
	Arabs	43	139	182	39	131	170	90.7	94.2	93.4
Gedera	Jews	73	38	111	27	14	41	37.0	36.8	36.9
	Arabs	5	47	52	4	43	47	80.0	91.5	90.4
Ein Ganim	Jews	247	116	363	95	50	145	38.5	43.1	40.0
	Arabs	14	28	42	14	27	41	100.0	96.4	97.6
Merchavia	Jews	32	95	127	24	4	28	25.3	12.5	22.0
	Arabs	58	58	116	10	30	40	17.2	51.7	34.5
Yessod	Jews	155	83	238	55	28	83	35.5	33.7	34.9
	Arabs	15	39	54	11	29	40	73.3	74.4	74.0
Ayeleth	Jews	50	0	50	3	0	3	6.0	—	6.0
	Arabs	8	12	20	4	6	10	50.0	50.0	50.0

In general the following relationships are indicated by our results:

(1) A continually high malaria incidence manifests itself by a much higher parasite rate among children and an equally high spleen rate among both adults and children. This condition is exemplified in the Arab villages Fejaz, Naaneh, Katra, Tiel and the Jewish settlements Katra, Yessod and Mishmar.

(2) In an area where malaria was highly prevalent and recently brought under control the blood rate is equally low and the spleen rate equally high among adults and children, or higher among adults, according to the duration of the control period. This condition is illustrated by Ein-Ganim, Nachlat Yehuda and Kinereth.

(3) In a place where malaria had been prevalent but had been under temporary control, or where a year of low prevalence is followed by an epidemic, both the blood and spleen rates are higher among children than among adults. Examples of this are Yavniel, Nehalal, Solim.

In places where there are no children the same correlation can be obtained between old settlers and recent immigrants. The underlying principle is the comparison of the blood and spleen rate of the susceptible, or new, and unsusceptible, or old, part of the population.

The conclusions to be drawn from this survey of over 5000 inhabitants of rural Palestine are:

(a) Taking the population as a whole, over 5 per cent. of the rural Jewish and over 15 per cent. of the rural Arabic population examined, actually carried parasites in their blood. Among the Jews the parasite rate in the Galilee settlements was a little below (4-5 per cent.), in Judea and Samaria a little above (6-7 per cent.) and in the Valley of Esdraelon more than double the average for the entire population. Among the Arabs the only notable exceptions which fell below the average were the village Hartuf and the Arabs living in the Jewish colony Yavniel; all the other villages gave a parasite incidence of 15-25 per cent. (The last figure is, however, higher than the average for the total population would normally be, because practically none of the adult females were examined.) The effect of so large a number of parasite carriers on the malaria prevalence is apparent.

(b) The blood and spleen rates taken by themselves indicate a high prevalence of malaria; but they are not a satisfactory indicator of the relative prevalence of malaria in adjacent communities, living under different conditions, and having different standards of life. In other words, there are other factors besides malaria prevalence which affect the blood and spleen rates of a community, which must be taken into account in the interpretation of the results of the examinations.

(c) More valuable information with regard to the malaria history of a place may be obtained from a correlation of the blood and spleen indices of the children and adults of the community, or of the new and susceptible and old and unsusceptible elements of the population.

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### 3. TREATMENT OF MALARIA.

BY J. M. SHAPIRO AND I. J. KLIGLER.

IN this country, as everywhere else, the treatment of malaria is the most mooted question. The common practice is to treat the febrile attack by intramuscular injections and leave the after treatment to the good will of the patient. The result is that the subsequent treatment is greatly neglected. At present most of the physicians here realise the importance of systematic treatment, but in civil practice it is extremely difficult to carry this into effect.

The presence of our malaria inspector in each of the demonstration areas made it possible to carry out systematic treatment of parasite carriers, under careful supervision, and to note the effect of such treatment on the occurrence of relapses. Most of the carriers under treatment were infected with *P. vivax*. Even the small number of individuals who were infected with *P. falciparum* at the examination, subsequently relapsed with the benign form of tertian malaria.

A uniform procedure was followed. The individuals who were to receive treatment were classified into two main groups:

- (1) Those who actually had one or another species of plasmodium in the blood.
- (2) Those whose blood was negative, but who had enlarged spleens, a history of a recent attack of malaria, or both.

Only one method of treatment was employed. All the adult patients were given 2 gms. of quinine sulphate daily, in three 10 grain doses, for a period of five days. Children received proportionate doses according to age. This part of the treatment was carried out under the direct supervision of a physician. Subsequent to that the malaria inspector administered 10 grains of quinine daily to each individual under treatment, for a period of one month. After that the patient was kept under observation and relapses recorded. It should be added that the quinine was in tablet form and was always given *per os*.

The treatment was started in February and March so that new infections could be almost definitely excluded during the first two or three months subsequent to treatment. The greater number of the cases were under supervision from six to eleven months.

The results are indicated in the table below (Table V). Altogether 358 people were treated. 260 of them actually had parasites in the blood at the time treatment commenced; the others either suffered from chronic malaria or had not received proper treatment at the time of the attack. All groups of cases showed a maximum percentage of relapses during the first four months following treatment, but relapses continued to appear to the end of the observation period. The total percentage of relapses varied according to

the group under treatment. Among the parasite carriers 30-40 per cent. of the cases relapsed. The highest incidence of relapses occurred in the group designated "Clinical Malaria." This group consisted of individuals suffering from chronic malaria which had already resisted other forms of treatment. The relatively low percentage of relapses among the cases in group 5, indicates that many of these cases had probably been cured by the previous treatment; at the same time the figures also show that many of the people who have a recent malaria history, are likely to be carriers, and should be treated as such, even though the blood and spleen are negative.

Table V.

*Treatment of Chronic Malaria.*

Type of carrier	Number treated	During or after treatment	Percentage relapses following treatment, Months after					Percentage cases without relapses, Months observed		
			1-2	3-4	5-6	7-10	Total	4-6	6-11	Total
<i>P. vivax</i>	213	3.3	9.4	8.9	3.8	4.7	30.0	4.7	65.3	70.0
<i>P. falciparum</i>	42	2.4	16.6	16.6	2.4	0.0	38.1	—	61.9	61.9
<i>P. malariae</i>	5	20.0	0.0	0.0	20.0	0.0	40.0	—	60.0	60.0
Clinical malaria	14	21.4	14.3	14.3	7.1	0.0	57.1	—	42.9	42.9
Malaria history*	26	3.8	7.7	3.8	0.0	0.0	15.4	11.5	73.1	84.6
Enlarged spleen	58	6.9	12.1	0.0	5.2	5.1	29.3	—	70.7	70.7
Total treated	358	4.8	10.6	8.1	3.9	3.7	31.1	3.6	65.4	69.0

\* Cases with history of an attack within four months prior to the examination; but blood and spleen both negative.

Table VI.

*Comparison of the Therapeutic Value of Quinine and Quinidine.*

Drug	Patient	Number treated	Percentage relapses following treatment		
			During or soon after treatment	1-4 months	Total
Quinine	Children	32	6.3	25.0	31.2
	Adults	21	5.0	19.0	23.9
	Total	53	5.6	22.7	28.5
Quinidine	Children	25	0.0	28.0	28.0
	Adults	20	10.0	15.0	25.0
	Total	45	4.4	22.2	26.6

The results obtained with this method of treatment are not as good as those reported by Kligler and Weitzman(4) who used the Bass or Standard Method(3). They do, however, compare favourably with those obtained by Anderson(2), using various methods of treatment. It seems to us that the important feature in the quinine treatment of malaria is the systematic administration of moderately large doses of quinine, over a long period (at least one month) subsequent to the treatment of the febrile attack. The methods may vary in detail without modifying the results, provided the general principle of systematic administration of the drug over a long period forms the basis of the method.

*Comparative Value of Quinine and Quinidine.* Acton(1) and his associates have reported results, which indicate that quinidine is as effective as quinine



in the treatment of malaria, particularly the benign tertian variety. We had occasion to test the effect of these drugs under comparable conditions in an epidemic which occurred in Hedera. The causative parasite was almost exclusively *P. vivax* and the patients were a group of children in an orphanage and a group of labourers. Each group was divided into two parts without selection. The form of treatment was that outlined above; one set receiving quinine sulphate, the other quinidine sulphate. The dosage is indicated below:

#### QUININE:

*Adults.* Five days 2 gms., thirty days 0.6 gm.

*Children.* 11-15 years, five days 1.2 gms., thirty days 0.5 gm.  
6-10 years, five days 1 gm., thirty days 0.3 gm.

#### QUINIDINE:

*Adults.* Five days 1.5 gms., thirty days 0.6 gm.

*Children.* 11-15 years, five days 1.2 gms., thirty days 0.5 gm.  
6-10 years, five days 1.0 gm., thirty days 0.3 gm.

The treatment was started in August, and the observations continued until the end of January: the results are tabulated on p. 297 (Table VI). Although the number of individuals observed is small, it is sufficient to indicate that quinidine is at least as satisfactory as quinine and is also perhaps a shade better, since smaller quantities of the drug yielded the same results as did the quinine treatment.

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#### 4. BREEDING PLACES AND TYPES OF ANOPHELES.

By I. J. KLIGER.

*Influence of Topography on Breeding Places.* In order to understand the nature of the breeding places in rural Palestine, it is necessary to be somewhat familiar with the topography of the country. So much has already been written on the topography of Palestine that it will suffice to sketch briefly those characteristics responsible for the breeding places and the malaria.

Topographically, Palestine is divided into four main divisions: (1) *The Coastal Plain*; a strip of land varying from four to twenty miles in width and extending from Haifa at the north to Gaza at the south. (2) At Haifa the Coastal Plain turns eastward into a plain extending from the coast of the Mediterranean to the valley of the Jordan. This plain is known as the Valley

of Esdraelon. It is a narrow strip of land, only a few miles in width which cuts the hills transversely and joins the Valley of the Jordan to the east with the Coastal Plain and the Mediterranean to the west. (3) Along the Jordan is the valley of the river extending from Lake Kinereth at the north, to the Dead Sea at the south. The greater part of this valley is less than a kilometre in width and lies in a ravine between two ranges of hills. (4) Between the Coastal Plain and the Jordan Valley, lies the range of hills which bisects the country longitudinally into two plains, distinct from each other in climate, habit, etc. These hills are chiefly of limestone formation. They rise slowly from the western and northern plains, through foot-hills, to the highlands of Judea, Samaria and Galilee; they then drop more sharply on the east to the Jordan.

This peculiar topography of the country and the seasonal rainfall account for the prevalence of malaria throughout Palestine. There are few large bodies of water. There are only three lakes (Kinereth, Merom and the Dead Sea), and a few narrow streams called rivers; but besides the Jordan they deserve that name only when they are filled by the winter torrents. The real causes of the malaria in the country are the seasonal rainfall, the limestone hills, and the sand dunes. The heavy winter rains which fall during December, January and February partly rush down the slope of the hills to the sea, and partly sink into them. The waters which rush into the plain run along winter wadis, eroding deep holes in their beds and giving rise to pools which remain all year. As the water approaches the sea it often finds its exit blocked by sand dunes, or outcrops of sandstone, and spreads out on the flat land, causing a series of extensive swamps at various points along the Coastal Plain. These swamps are known as the Coastal Marshes. The waters which penetrate into the hills, crop out at the foot-hills in the form of numerous springs which run along in overgrown, neglected, creeks, or wadis, for greater, or lesser, distances, according to the volume of water issuing from the spring. On the top of the hills the inhabitants supply the lack of natural storage by building cemented cisterns which catch and store the rain water.

The sources of the mosquitoes and the consequent malaria in the various regions of the country—hills, foot-hills and plains—are thus obvious. In the hills, *e.g.* Jerusalem, Safed, etc., the cistern is the habitat of the anopheles and is the principal cause of malaria. In the foot-hills the numerous springs (only few of which are utilised), and their overgrown wadis constitute the chief source of trouble (districts of Ekron, Merchavia, Nuris, Beisan, Yavniel, etc.). In the flat plain, along the coast, the erosion holes in the wadi beds tapping the ground water, and the waters blocked by sand dunes (Kishon, Cabara, Rubin), or obstructions caused by sandstone along the sea (Athlit, Tantura, Atta in Hedera), constitute the breeding places of mosquitoes and the sources of malaria.

Aside from these breeding places there is one bad swamp of great magnitude—the Huleh Marshes above Lake Merom. This marsh is the worst and

most extensive in Palestine and makes the surrounding plain at the headwaters of the Jordan and Lake Meron uninhabitable.

*Classification of Breeding Places.* Most of the anopheles breeding places are due either to century old neglect by man, or to man's carelessness. There are the numerous springs mentioned above, with an abundance of water which runs to waste and forms overgrown streams (perennial wadis), and swamps, offering all kinds of ideal conditions for anopheles breeding. There are also numerous watering holes and primitive leaky irrigation ditches, which are responsible for a goodly portion of the malaria in Palestine. Large swamps in the true sense of the word exist as indicated above only in Huleh, in the low flat areas of the Jordan and in the Coastal Plain.

For convenience the principal breeding places have been grouped into four categories. These are given below, approximately, in the order of their relative importance, in so far as the causation of malaria is concerned:

- (1) Uncontrolled springs and seepage areas, and their resulting wadis.
- (2) Irrigation canals.
- (3) Accumulation of stagnant rain or ground water, holes in winter wadis, etc.
- (4) Breeding places along receding shores of natural streams and lakes and natural low lying swamps.

(1) The breeding places of the first category are found in practically all of the settlements located in the foot-hills. There is always a spring, or a larger, or a smaller seepage area (series of springs) which is partially or wholly uncontrolled. The excess water runs off in a wadi, seeps through gravel beds and zigzags for long distances; the wadi is shallower in some parts and deeper in others, is badly overgrown along its whole length and presents a variety of excellent breeding places more or less difficult to control.

(2) The irrigation canals present a serious problem. They are in reality poorly constructed ditches, too shallow to hold all the water they carry, and too pervious to exclude seepage along their entire length. In a short time the ditch itself becomes overgrown. The long, overgrown, open ditch, the swampy areas resulting at low lying points from seepage through the walls, and the marshes caused by the overflow of the canal, give rise to a large number and variety of breeding places. This problem has become more serious recently, since almost everywhere irrigation is being employed more extensively for vegetables, tobacco, etc.

(3) Stagnant accumulations of water exist almost everywhere, but are particularly abundant in the highlands and in the plain near the coast. These accumulations take the form of cisterns, wells or reservoirs, watering holes along dry wadis, deep erosion holes in winter wadis tapping ground water, accumulations of rain water which find their way to the sea blocked by sand dunes or other obstacles and others of similar character. Inland they are usually small and easily controlled by cleaning and oiling, but near to the

coast they assume the characteristic appearance of the coastal swamps caused by the sand dunes, or sandstone outcroppings, and present serious difficulties. The Cabara swamps, the old Athlit swamps, Birket Atta, are examples of this type of breeding place.

(4) The breeding places of the fourth class are practically limited to the Huleh swamp, Lake Merom and the Jordan. They are a serious menace, and can only be got rid of by drainage.

From the standpoint of control we may say, by way of anticipation, that malaria caused by the breeding places of the first two and some of those belonging to the third categories can be readily controlled, and will be completely eradicated as soon as the level of intelligence of the native population is raised, and as soon as the country is so well developed that all the spring waters are properly and intelligently exploited. The large coastal marshes, however, can only be controlled by radical drainage. One of these swamps at Athlit, has already been drained by a company which has received a salt concession and the I.C.A. has agreed to drain the largest of them, the Cabara swamp, on the basis of a land concession given them by the Palestine Government.

*Types of Anopheles.* Eight species of anopheles have been found in Palestine. One, *A. bifurcatus*, is mainly a cistern breeder, and is found almost exclusively in urban centres. *A. hyrcanus* and *A. algeriensis*, are swamp dwellers which do not enter houses and are, therefore, not to be considered as malaria vectors. *A. pharoensis*, the Egyptian anopheles, is very rare in Palestine. *A. multicolor*, breeds only in salt water and its distribution is, therefore, fairly limited. This leaves three important rural mosquitoes all of which are widely distributed and known to be malaria vectors. These are: *A. elutus* (*maculipennis*), *A. superpictus* (*palestinensis*), and *A. sergenti* (*culicifacies*).

*Character of Breeding Places.* The character of the swamp usually determines the type of mosquito which will breed there. This is at times so clear cut that one can almost predict with certainty from the character of the swamp the types of anopheles and *vice versa*. Stagnant pools in winter wadis, stagnant water overgrown with ranunculi, wells and reservoirs overgrown with algae invariably breed *A. elutus*. The partially stagnant swamps caused by overrunning irrigation ditches and streams, breed either *A. elutus*, or *A. sergenti*, or both. Very slowly moving streams, such as open seepage canals and sluggish parts of wadis, seepage under rocks and pebbles, etc., will almost invariably harbour *A. sergenti* (*culicifacies*); at times they may also be associated with *A. superpictus*. Steadily flowing streams, emanating from springs, breed almost exclusively *A. superpictus*. Salt water, containing from 1-3 per cent. salt, harbours only *A. multicolor*. In sheltered and badly overgrown, stagnant, muddy swamps, one finds *A. hyrcanus* or *A. algeriensis* or both. *A. bifurcatus* is exclusively a cistern breeder, although Buxton found it once breeding out of doors. *A. pharoensis* is rare in Palestine, but

## DESCRIPTION OF MAP.

*Rosh-Pinah Area.* Population 2849. Area 69.4 sq. kil.

	Index: Blood	Spleen	
		Adults	Children
Jews	2.5 %	28.3 %	33.7 %
Arabs	16.0 %	65.2 %	68.6 %

*Yavniel Area.* Population 1385. Area 98.8 sq. kil.

	Index: Blood	Spleen	
		Adults	Children
Jews	2.1 %	12.7 %	23.9 %
Arabs	1.3 %	—	—

*Kinereth Area.* Population 915. Area 22.6 sq. kil.

	Index: Blood	Spleen	
		Adults	Children
Jews	4.2 %	30.7 %	20.9 %
Arabs	—	—	—

*Merchavia Area.* Population 1420. Area 87.6 sq. kil.

	Index: Blood	Spleen	
		Adults	Children
Jews	6.3 %	20.9 %	19.0 %
Arabs	25.8 %	17.2 %	51.7 %

*Zichron-Jacob Area.* Population 2186. Area 80.6 sq. kil.

	Index: Blood	Spleen	
		Adults	Children
Jews	18.1 %	42.0 %	—
Arabs	—	—	—

*Ein-Harod Area.* Population 2425. Area 51.8 sq. kil.

	Index: Blood	Spleen	
		Adults	Children
Jews	8.8 %	25.0 %	—
Arabs	—	—	—

*Hedera Area.* Population 1390. Area 49.2 sq. kil.

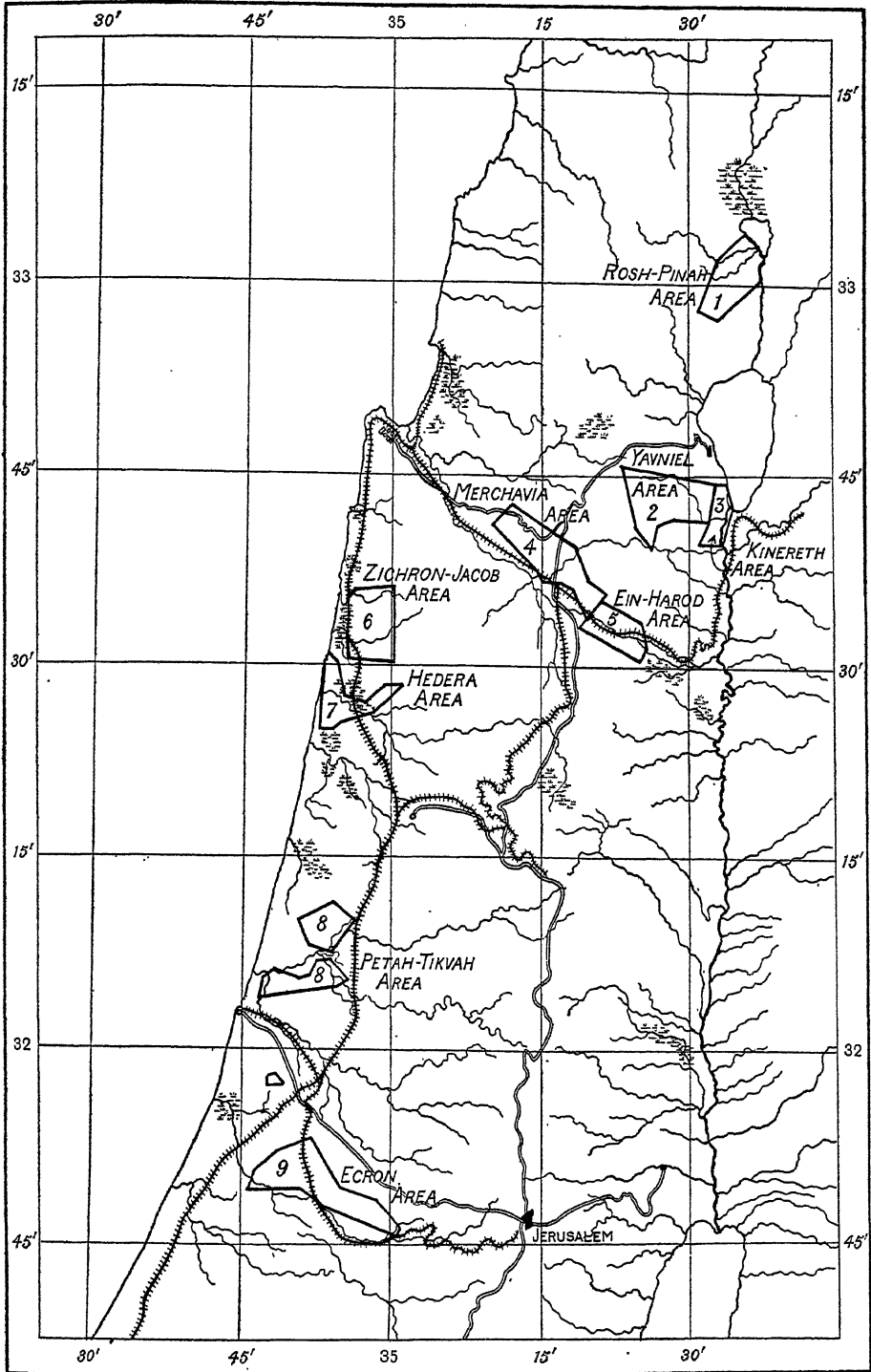
	Index: Blood	Spleen	
		Adults	Children
Jews	5.6 %	51.9 %	14.6 %
Arabs	—	—	—

*Petah-Tikvah Area.* Population 4990. Area 67.2 sq. kil.

	Index: Blood	Spleen	
		Adults	Children
Jews	5.7 %	33.2 %	42.2 %
Arabs	19.0 %	100.0 %	96.4 %

*Ecron Area.* Population 4519. Area 106.1 sq. kil.

	Index: Blood	Spleen	
		Adults	Children
Jews	5.7 %	19.8 %	22.9 %
Arabs	18.2 %	69.6 %	82.4 %



we have found it breeding in the Cabara swamp where *A. elutus* is usually found.

*Distribution of the Various Types of Anopheles.* The anopheles are not uniformly distributed throughout Palestine; this difference in distribution depends not on climate but on the character of the breeding places prevailing in the various districts. Thus in Ein Ganim, Hedera, and elsewhere in Judea and Samaria, where the wadis shortly after the rains present the appearance of a series of isolated stagnant pools, *A. elutus* are found; while in wadis Melat and Musrara, also in Judea, which are fed by springs and consequently have a continuous slow-moving stream, *A. superpictus* and *A. sergenti* are found.

In Judea both *A. elutus* and *A. superpictus* are found, but the latter predominates where the breeding places are caused by springs (Ekron, Hartuf, Ramat Gan.), and the former is practically the only house visiting anopheles where the breeding place is a winter wadi (Ein Ganim).

In Samaria (Hedera Area), along the coast, *A. elutus* is practically the only house visiting anopheles; but *A. superpictus* is occasionally found and, rarely, also *A. pharoensis*.

In Phoenicia (Zichron Jacob and Athlit), *A. elutus* is the dominant type, but *A. multicolor* is found in the salt marshes, and *A. superpictus* in the spring wadis. The latter are, however, uncommon in comparison with *A. elutus*.

In the Valley of Esdraelon, *A. elutus*, *A. superpictus* and *A. sergenti* are all equally abundant. The former are more prevalent in the spring and late fall, and the last is most numerous in the late summer and fall.

In Lower Galilee, *A. superpictus* and *A. sergenti* are practically the exclusive species in the Yavniel District, where the only breeding places are the open seepage canals and flowing wadis. Along the Jordan and the Sea of Galilee *A. elutus*, *A. sergenti* and *A. superpictus* are equally common. *A. elutus* breeds along the shores of the Jordan, in the beds of ranunculi, or in the swampy areas where the low ground is flooded by the Jordan. *A. superpictus* breeds in the flowing wadis, and *A. sergenti* is found in the seepage from the irrigation canals and under pebbles and rocks in the wadis or in the Jordan.

In Upper Galilee (Rosh Pina Area), *A. elutus* is the principal species, but *A. superpictus* is commonly found. In the late summer and fall *A. sergenti* is also found but in relatively small numbers.

Occasionally one also finds the urban mosquito, *A. bifurcatus*, which breeds in cisterns. This mosquito was found by us in Hartuf (Ekron Area) and in Poria (Yavniel Area). We have never found it breeding out of doors.

*Seasonal Prevalence.* *A. superpictus* and *A. elutus* begin to breed at the end of March, or early in April, and continue throughout the year, until the beginning of the heavy rains at the end of November, or sometime in December. From about the middle of August to the middle of October, there is a decided diminution in the numbers of *A. elutus*, but at the latter period there is again a sharp increase. The season of activity of *A. superpictus* follows closely that

of *A. elutus*, but there is no increase in their numbers in the fall as is the case with *A. elutus*. The active season of *A. sergenti* is not as yet well defined, but its larvae usually become evident in July or early in August and the adult remains the dominant mosquito throughout September and October. After that they are equally abundant with *A. elutus*. Breeding continues until the end of December and in warmer places even into January. The exact cause of this seasonal fluctuation is not clear, but there seems little room to doubt that it is chiefly a temperature relation. *A. algeriensis* breeds actively between November and June, while *A. hyrcanus* breeds more actively throughout the spring and summer months.

*Active Period of Anopheles.* There is no true hibernation in Palestine. The adult anopheles may be said to be active throughout the year. During the months of January to March one frequently finds anopheles with freshly ingested blood; while in the laboratory we have observed them laying eggs when the room temperature was between 16° and 17° C. *A. elutus* and *A. superpictus* do take winter quarters (store-houses and cellars), during the latter part of November and December; the prevailing low temperature (10–12° C. in the plains) throughout the winter, also greatly inhibits their activity. However, between rainy days there are often periods when the temperature rises to 16° and 18° C. and the anopheles may then resume activity. This accounts for the sporadic cases of primary infections which actually occur during the winter months. We are not certain as yet whether *A. sergenti* also takes winter quarters; thus far we have not found them.

The immature stages of all house visiting anopheles, excepting *A. bifurcatus*, are inactive during the winter. At no time during the months January to March were larvae of *A. elutus*, *A. sergenti*, *A. superpictus*, or *A. multicolor* encountered. During these months *A. algeriensis* is readily found breeding outside and *A. bifurcatus* in cisterns. This holds true at any rate for the plains and foot-hills. In the highlands breeding apparently ceases entirely; while in the Jericho region, where the climate is semi-tropical, Buxton reports breeding of *A. sergenti* throughout the winter period.

During the months of April to December anopheles are active in all stages. The larvae appear late in March and early in April and disappear after the first heavy rains, which usually come late in November, or early in December. Adults appear about the middle or end of April, and enter winter quarters in November and December.

*Flight of Mosquitoes.* We have performed no experimental flight tests; but have ample evidence that both *A. sergenti* and *A. elutus* have a much greater range of flight than is commonly supposed. In Hedera, *A. elutus* travelled a distance of 2·4 kilometres and caused a severe epidemic. In the Ekron district the Melat swamp has been proved to be the cause of malaria in Naaneh and Ekron, both two kilometres or more away. In Balfouria, *A. elutus* travelled from a well more than two kilometres away. In Beth-Alpha, *A. sergenti* came from a swamp 2·5 kilometres from the settlement.



These observations are definite, because no other breeding places could be found closer to the settlement to account for the mosquitoes and, with the control of the suspected breeding places, the mosquitoes disappeared.

An important fact regarding the flight of mosquitoes, generally overlooked, is that they do not come when the wind is strong, but when it is gentle, or when it has subsided. While strong winds are blowing the mosquitoes seek shelter and are not active. The range of flight, in so far as local anopheles are concerned, seems to be determined by factors which appear to us more important than the wind. The distance of flight depends on the number and density of settlements near the breeding places and on the intensity of breeding. In other words, the distance of spread from a breeding focus varies directly with the intensity of breeding and inversely with the density of the settlements and population. If there are no settlements near the swamps, or if the breeding is very heavy, the mosquitoes travel relatively long distances in order to obtain their food.

*Relative Importance of the Various Anopheles as Malaria Vectors.* Happily not all of the anopheles mosquitoes enumerated above are of equal importance as malaria vectors. We have not, as yet, sufficient information on this subject. It is fairly certain, however, that *A. hyrcanus* and *A. algeriensis* which are not house visitors, are negligible in so far as malaria transmission is concerned. *A. bifurcatus* is of great importance in the urban and semi-urban highland districts which depend on cisterns for their water supply; but it is relatively of little significance in the rural communities of the foot-hills and plains which derive their water supply from springs or wells rather than from cisterns. *A. multicolor* is a salt water species of comparatively limited distribution and, judging from epidemiologic evidence, is not seriously concerned in the spread of malaria. All of the three remaining common species of anopheles are probably important vectors, although *A. elutus* and *A. sergenti* appear from epidemiologic evidence to be of greater importance than is *A. superpictus*. Our experience in places having only *A. superpictus* (Ramat-Gan and Ayelet) was that there was very little malaria even when mosquitoes were abundant. Against this experience, however, is that of various other workers, and the fact that we have found an infected specimen in our dissections, which indicates that it is an important vector.

*Incidence of Infected Mosquitoes.* The number of dissections made in our laboratory thus far are not sufficient to warrant a definite conclusion with regard to the incidence of infected mosquitoes in nature. Enough work has been done, however, to show that the incidence is not higher than that found in Macedonia or other malarious areas. From October to the end of January we have dissected 630 *A. elutus* (*maculipennis*), 294 *A. sergenti* and 39 *A. superpictus*. Seven *elutus* and one *superpictus* were found infected, giving an incidence of 1.1, 0.0 and 2.6 per cent., respectively. The incidence of infection among the *A. elutus* for October, December and January were 1.3, 1.6 and 0.85 per cent., respectively.

*Infectivity of the Local Anopheles.* A limited number of infection experiments have also been carried out. These have demonstrated that *A. elutus* can be infected with either *P. vivax* or *P. falciparum*, contrary to the suggestion recently made by Buxton that this mosquito carries only the parasite of benign tertian malaria. Under the conditions of our experiments, that is with single feedings, we failed to infect mosquitoes when fed on patients taking quinine, irrespective of whether the gametocytes were of *P. vivax* or of *P. falciparum*. The number of experiments is, however, small and these findings cannot, therefore, be considered as conclusive.

#### SUMMARY.

In this section we present briefly the main sources of anopheline mosquitoes, and describe the habits of the species more or less commonly found in Palestine. It is shown how the peculiar topography of the country, the limestone hills, the seasonal rainfall and the sand dunes lead to the formation of characteristic breeding places. Depending on the character of the breeding place one or all of the three common house visiting anopheles are widely distributed throughout rural Palestine. Two of these mosquitoes, *A. elutus* and *A. superpictus*, have thus far been found infected in nature, and the former has also been infected experimentally with *P. falciparum*, as well as *P. vivax*.

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### 5. MALARIA CONTROL DEMONSTRATIONS.

BY I. J. KLIGLER AND J. M. SHAPIRO.

IN 1921 Kligler and Weitzman conducted malaria control demonstrations in two selected areas in the region of the Jordan and Lower Galilee. These demonstrations indicated the possibility of controlling malaria in those areas at a reasonable cost. It was desirable, however, to extend these studies and to determine the possibility of large scale malaria control in Palestine, at a moderate cost. One of the tasks, therefore, of the Malaria Research Unit, which was attached to the Palestine Health Department on Sept. 15, 1922, was to organise a number of malaria control demonstrations with this object in view. Tentative work was commenced immediately, but the organisation was not completed until the beginning of 1923.

*Demonstration Areas.* With the knowledge of the general topography of the country, the character of the breeding places, and the prevalence of malaria as a basis, nine malaria control demonstrations were organised in different parts of the country, including all varieties of topographic conditions (excepting highland settlements), population, etc. Table VII (p. 314)

contains a summary of each area; populations, blood and spleen indices, incidence of malaria, and degree of control and costs.

*Organisation.* The demonstrations were organised as a unified scheme, under central direction. A specially trained anti-malaria inspector responsible for the carrying out all of the control operations, was placed in each demonstration area. The inspector lived in his district and had complete supervision of the breeding places, actual and potential. There were also, at first, two senior inspectors (later reduced to one), who visited the various districts from time to time, made thorough inspections, gave special instructions to the local inspectors and reported on the general situation and the effectiveness with which the work was carried out. The Field Medical Officer and the Controller of the Unit also visited the various areas from time to time, for special inspections and surveys, or when special problems arose. The field inspectors were required to send weekly reports, in the form of diaries, indicating concisely and clearly their daily activity, the breeding and malaria conditions, and the daily progress of the work. In this way the work was closely knit and the central office kept in constant touch with the work in the field.

*Area.* The total area under control amounted to over 600 square kilometres. The extent of the individual demonstration areas varied considerably, since the size of the areas was limited by the ability of a single inspector to carry out effective control of the breeding places. If the number of breeding places was large the area was of necessity small and *vice versa*.

*Population.* The population included in the Control Areas amounted to 21,230, of which 13,580 were Jews and 7650 Arabs. In other words the Control Areas contained approximately 5 per cent. of the total rural population and 93 per cent. of the rural Jewish population. All classes of population were included in the Demonstration Areas. There were old Jewish settlers in *Judea* and *Galilee*, who were saturated with and more or less resistant to malaria: and the newer settlers in the *Valley of Esdraelon* also saturated with malaria, but not as yet for a sufficient length of time to have acquired any resistance to the disease. There were included the crowded native villages with their backward inhabitants, as well as the more modern European settlements or colonies. This heterogeneous population naturally complicated the problem; some elements were more responsive and more easily influenced, others less so. On the whole, however, all sections of the population manifested some interest in the work, once the native suspicion was allayed.

#### METHODS OF CONTROL.

Our efforts to control malaria were directed almost entirely towards eradication of anopheles mosquitoes, but the fight against the parasites was also employed as an aid in the campaign. The general plan of campaign in any given demonstration area consisted in the following steps: (a) a complete survey of the breeding places within a radius of two kilometres or more from

the settlements; (b) a survey of the parasite and spleen rates, followed by intensive treatment of carriers; (c) systematic house to house search for and destruction of hibernating mosquitoes; (d) control of mosquito breeding by various devices to be described below. The first three steps were completed during the months of January to March, when there was no breeding. They were preparatory in nature, paved the way for the control work, and awakened public interest. The actual campaign was started in April with the commencement of anopheles breeding and continued until the heavy rains, which were unusually late this year, and did not come till late in December.

*Quinine Prophylaxis as a Control Measure.* Since the war the value of quinine as a malaria control measure has been the subject of a great deal of controversy. Most of the people who have had direct experience with quinine prophylaxis during the war are of the opinion that it is worthless. Our own experience in 1921<sup>(2)</sup> and 1922<sup>(3)</sup> has made us very sceptical as to the value of this measure as a method of control. In 1921 we have seen that even the fairly careful daily distribution of 0.6 gm. of quinine sulphate did not prevent the appearance of new cases of malignant malaria. In 1922 we had definite evidence that quinine merely prevented the appearance of symptoms, but that the infection remained latent, and so soon as prophylactic quinine stopped either relapses or primary attacks occurred.

There are other considerations which make it undesirable, except under circumstances where there is no other alternative, to rely on quinine as a method of control. Aside from the fact that the drug is relatively expensive it is always very difficult to organise systematic distribution of quinine, and even with the best organisation it is practically certain that a considerable proportion of the population fail to take the prophylactic regularly. With careful control and supervision, consisting of daily individual distribution of quinine, we could never be sure that more than 75 per cent. of the people actually took the quinine with any degree of regularity.

As a result of this experience it was decided to rely very little on quinine prophylaxis in the control of malaria. Regular quinine prophylaxis was not given anywhere. Quinine was used only as a temporary measure where mosquito control had broken down. Under these special conditions intensive quininisation in therapeutic doses, 2/3 gm., was employed during the period of prevalence of mosquitoes, and stopped soon after breeding was again under control and the adult anopheles index reduced to a safe margin. Used in this way quinine proved an effective aid in malaria control. But even with these large doses there were, in Kinereth, six primary cases within a week after the quinine distribution had been discontinued.

#### CONTROL OF MOSQUITO BREEDING.

*Types of Breeding Places in the Control Areas.* It would take us too far afield if we were to describe in detail the various breeding places encountered. In general the swamps found in the various control areas conformed to types

as described above. In the *Ecron* area which is in the foot-hills of Judea we had to deal mainly with springs and seepage wadis and pits dug by shepherds along dry wadi beds. In the *Petach Tikva* area, in the Plain of Sharon, we encountered the problems of erosion holes in winter wadis and of the numerous wells and reservoirs used in the irrigation of the orange groves. In both these areas control of breeding was comparatively easy and many of the important breeding places can be entirely eliminated at a relatively small sum.

In the *Hedera* area, which is in the Coastal Plain in the district of Samaria, the problem was more difficult. Here, we had to deal with all three types of swamps. In the settlements along the foot hills (Karkur, Gan Shmuel) there are seepage areas and spring wadis. In *Hedera* proper, there are the erosion holes in the winter wadi and a coastal marsh caused by sandstone obstructions. It was possible, by the various methods to be described below, to limit breeding in the wadis, but the coastal marsh remained out of control and was the cause of a serious epidemic in Hedera. Plans are under way to bring this marsh under control, by arranging to pump out the water for irrigation purposes.

The *Zichron* area, in the district of Phoenicia, also lies partly in the Coastal Plain, and presented problems similar to those encountered in Hedera. Control was effected in the wadis and irrigation canals, but we were helpless in so far as the extensive coastal marsh, Cabara, was concerned.

In the *Merchavia* and *Nuris* areas in the Valley of Esdraelon the chief problems were spring wadis, irrigation canals, and watering holes. The Valley of Esdraelon presents a unique picture. It is a narrow strip of land lying between two ranges of hills. At the foot of these hills, on either side of the valley, there are numerous springs, the waters of which, not being utilised for better purposes, spread out in winding wadis, flood low lying areas, and give rise to extensive swamps. Owing to the large number of these neglected springs the valley ranks among the most malarious regions in the country.

The ease, or difficulty, with which the control of these swamps was effected depended largely on the number of springs, or the extent of the seepage areas. As the number of settlements in the valley increases, more of the springs are being utilised, their streams regulated and the swamps caused by them eliminated. This year a considerable number of springs have been collected and brought under control in the Ein Harod and Merchavia areas, and large tracts of swamps have been dried. Part of the water from these springs is to be used for drinking and irrigation purposes, and the rest is run off in closed subsoil drains.

In the *Yavniel* district, the control depends entirely on the regulations of springs and their wadis, and the irrigation canals. These can easily be taken care of, provided the population is willing to co-operate. In most places this is the case and the results are quite satisfactory. We have been able to keep this district fairly free from malaria.

The problem of control in the *Kinereth* district centres in the Jordan and

a few spring wadis. The greatest efforts had to be concentrated on the Jordan; with a good deal of hard work the breeding could be checked sufficiently to prevent the outbreak of any serious epidemics.

The *Rosh Pina* district may be divided into two parts. The section around Rosh Pina obtains its mosquitoes from seepage wadis, which can be readily controlled. Another group of villages are located around Lake Merom and the Huleh Marshes. There it is impossible to do anything against mosquito breeding and mechanical protection and quinine are the only protective palliatives available.

*Methods of Control of Mosquito Breeding.* The methods used to check breeding varied with the character of the breeding place. Whenever possible we resorted to drying. For this purpose two simple devices were utilised which proved very useful in Palestine, and may perhaps also be of use elsewhere: (1) Wadis originating from a single spring or spring head were dried intermittently by damming the stream near its origin and releasing the water every 5 to 10 days, according to the rate with which the basin behind the dam was filled. (2) Irrigation ditches and their swamps, wadis on flat areas which could not be dammed, and open drainage canals were dried by alternating or periodically deflecting the flow in another direction. In this way extensive breeding areas could readily be controlled with little effort and usually with only a small initial outlay. These methods were used in several demonstration areas (Zichron, Merchavia, Nuris, Yavniel and Kinereth) with excellent results.

In pools, erosion holes in winter wadis and similar accumulations of water, we resorted to the cleaning of vegetation with or without the subsequent use of a larvicide, according to the need. As a rule there was little or no breeding in clean pools free from vegetation.

We conducted an extensive series of experiments with various mixtures of kerosene and crude oil, with and without the addition of small quantities of vegetable oils and oleic acid. We also experimented with various mixtures of cresol. The larvicides which proved most useful for our purpose were: (1) a paraffin-crude-oil mixture containing 0.1 per cent. castor oil, (2) Paris Green, (3) a water solution of cresol.

After much experimentation we found that for practical purposes the best kerosene mixture consists of nine parts paraffin (kerosene) and one part heavy commercial crude-oil, to which 0.1–0.2 per cent. castor oil was added. The film produced by this mixture has greater penetrative power than that of paraffin alone, is more stable and most durable. The castor oil (4) greatly increased the spreading and penetrating power of the mixture and at the same time does not affect the tensile strength of the film. This mixture is also more economical since 10 c.c. are ample for one square metre, whereas 25 c.c. of paraffin alone or paraffin and crude-oil are required for the same area.

Paris Green proved particularly useful in overgrown pools and wadis, where paraffin (kerosene) mixtures are practically useless. The Paris Green

was diluted with road dust or fine sand in the proportion of one part Paris Green to 100 parts of diluent and employed in the manner described by Barber<sup>(1)</sup>. We found 10 gms. of the arsenical quite sufficient for 100 square metres of swamp. In other respects, such as the failure to affect culex larvae, the results were the same as those reported by Barber. We also found that anopheles eggs and pupae were not killed by the arsenical, and that consequently the powder had to be applied every 7 instead of every 10 days as in the case of oiling.

In the open irrigation wells and reservoirs in the orange groves we experimented with cyprinidons and with copper sulphate. The chief difficulty in these places are the algae, which grow rapidly and in abundance and provide the necessary shelter for anopheles larvae. Such wells when free from algae rarely breed. Control of breeding depends on the success with which the algae themselves are destroyed or their protective effect counteracted. Copper sulphate in a dilution of 1 : 1,000,000 was sufficient to destroy the algae and keep the tank free from vegetation for three to four weeks. The experiments with cyprinidon were not conclusive. They proved useful in some tanks, but in others they fell a prey to other fish, turtles, or water snakes, and restocking was required. It is too early as yet to say which of these methods is going to prove most useful under local conditions, but the experiments are being continued. We have also experimented with *Gambusia*, brought from the United States, but these experiments have not as yet passed the aquarium stage. So far our attempts to stock ponds have failed.

*Factors complicating Malaria Control Work.* There are several factors which make control of malaria in Palestine more difficult than it would ordinarily be. One is the nomadic Bedouin, the shepherd is another, and the primitive irrigation system a third. Early in the spring the nomads come in with their flocks from the east in search of pasture and water. They move across the plains, settling along wadis, changing camps with changing conditions, rarely remaining in one place all summer. Before the onset of the rainy season they return in the direction from which they came. These tribes are, as a rule, heavily infected with malaria; we have found among them parasite indices as high as 25 per cent. and spleen indices of 80 per cent. or over. Moving along the plains and living close to marshes they increase the incidence of infected mosquitoes and leave a trail of infection behind them, coming and going. Aside from that they continually create new marshes by damming up streams to facilitate the watering of their cattle or, if they are also semi-agricultural tribes, for primitive irrigation. Their cattle trample the banks of the creeks and before long the regular trimmed banks of the stream are converted into a hoof-marked bog, ideal for breeding of *A. sergenti* or *A. elutus*.

The shepherds complicate the problem in two ways. Like the Bedouin tribes they destroy the banks of regulated streams. Where there are no streams they dig primitive wells or watering holes along beds of dry wadis,

for watering their flocks. These holes furnish excellent breeding places for *A. elutus*. We have had several small outbreaks of malaria, which were attributable to such holes opened in out of the way places and without knowledge of anyone.

The primitive irrigation canals are also the source of a great deal of worry. They are either too shallow for the amount of water they carry, or the walls leak or the water is too sluggish. Whether the fault be at one point or another they always breed and are always a serious source of trouble.

*Result of the Control Measures.* The effect of the control measures can best be seen from Chart 1, showing the malaria incidence in the control areas in 1922 and 1923. The difference in the malaria incidence for these years is brought out more effectively in Chart 5 and Table VII, giving the average monthly incidence for the period prior to control (where control was started in 1921 or 1922) for 1922 and 1923, respectively. It is evident that, excepting the Hedera and Rosh Pina districts, there was a decided decrease in the malaria incidence everywhere in comparison with 1922 as well as with the period prior to control.

In 1921 demonstrations were conducted in the districts of Kinereth, Menachemia and Yavniel. During that year malaria was reduced in Kinereth from an average monthly incidence of 9.4 to 1.4 per cent., in Menachemia, from 5.5 to 0.84 per cent. and in Yavniel from 4.2 to 0.4 per cent. In 1922 these demonstrations were continued with somewhat less intensity, and similar demonstrations were started in the Ecron and Petach Tikwa areas, in Judea.

In the Judea areas there has been a steady decrease in the malaria incidence from the time control began. During 1923 there were practically no primary infections, and relapses were steadily becoming fewer and fewer. The extremely low rainfall in the winter of 1922 and 1923 greatly facilitated the control work.

In the *Hedera* district steady progress was made in 1923 until there came an explosive epidemic in Hedera itself, caused by an uncontrolled coastal marsh (Birket Atta)  $2\frac{1}{2}$  kilometres from the settlement. This marsh was uncontrollable, and moreover we considered it beyond the normal flight of mosquitoes. Plans are now effected to control this marsh at a moderate cost and its control should enable us to bring about a considerable reduction in the malaria incidence in this area in 1924.

The *Zichron* area is skirted by the Cabara swamp, the largest of the Coastal Marshes which lies within 1.5 to 2.0 kilometres from the settlements. There, control of mosquito breeding was out of the question. The marked reduction in the malaria incidence in this area was achieved by three measures: (1) The reduction of the number of carriers by intensive treatment. (2) The elimination of breeding in the vicinity of the settlements. (3) The systematic destruction of adult mosquitoes in the houses. Despite the noted improvement it is evident that the elimination of malaria in this area is not possible



by these half measures. This result can be achieved only when the Cabara swamps are completely drained.

In the *Valley of Esdraelon* the results were very encouraging. Despite the drainage operations which were going on in all the settlements, and despite the numerous potential breeding places, breeding was kept at a minimum, and the malaria incidence, particularly the incidence of primary infections,

Chart 5. Comparison of mean monthly incidence of Malaria prior and subsequent to control.

CHART No. 5  
D E M O N S T R A T I O N   A R E A S

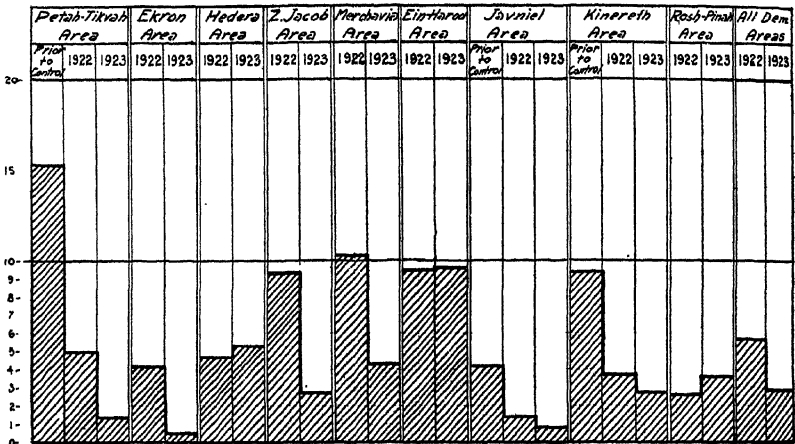


TABLE No. VII

Demonstration Areas	Petah-Tikvah			Ekron			Hadera			Z. Jacob			Mercharia			Ein-Harod			Yavniel			Kinereth			Rosh-Pinak			All Dem Areas			
Year	Prior to Control	1922	1923	1922	1923	1922	1923	1922	1923	1922	1923	1922	1923	Prior to Control	1922	1923	Prior to Control	1922	1923	Prior to Control	1922	1923	1922	1923	1922	1923					
Average monthly Population		497	619	1085	714	2385	698	922	1548	1535	448	935	448	800	440	957	1038	319	454	467	820	875	6758	10042							
Average monthly Cases		76	31	15	30	12	33	49	144	41	46	40	47	77	18	13	8	30	17	13	21	31	382	286							
Average monthly Incidence		15.3	5.0	1.4	4.2	0.5	4.7	5.3	9.3	2.7	10.3	4.3	9.5	9.6	4.2	1.4	0.8	9.4	3.7	2.8	2.6	3.6	5.7	2.9							
Percent of Cases Prior to Control	1923	+90.9															+81.0			+70.3											
	1923	+72.0			+88.4			-12.8			+71.0			+58.6			-1.1			+43.0			+24.4			-38.6			+49.0		
	1922																														

\* This incidence is based on approximate figures from June to December, since full reports were not available.

was much lower than in 1922. At first sight it appears that in the Ein Harod area there was an increase in the malaria incidence. This increase is, however, only apparent since the bulk of the cases were relapses among those who had attacks during the 1922 epidemics. In so far as primary cases are concerned there was a decided reduction over 1922.

In 1922 the results of the control work in the *Kinereth* and *Yavniel* areas were not as satisfactory as they were in 1921. But in *Kinereth* the mean monthly incidence in 1922 was still only 3.7 per cent. as against 9.4 per cent. prior to control, and in the *Yavniel* area the average incidence for *Yavniel*,

Bet Gan and Poria in 1922 was 1·7 per cent. against 4·2 per cent. for the same places during the ten months period before the control work began. In 1923 there was a further reduction in both the Yavniel and Kinereth areas as compared with the incidence of 1922, but the incidence still remained higher than in 1921. We believe that with a little more intensive work the incidence in both places can be brought down to the level reached in 1921.

In the *Rosh Pina* area control began in 1921 and malaria was practically absent in the group of villages near Rosh Pina. This condition continued until the month of November 1923, when there was a sudden influx of *A. elutus* from an unknown source. This district and that of Hedera are the only two which showed an increase in the incidence over that of 1922.

Table VIII.

*Cost of Control Operations in the Demonstration Areas, 1923.*

Demonstration Area	Population	Salary and travel*	Supplies	Casual labour and misc. ex.	Total	Per capita
Ecron	4519	P 24695·0	1601·0	446·5	26742·5	5·9
Petach Tikwa	4990	37555·0	2474·5	431·0	40460·5	8·1
Hedera	1390	22145·0	3750·4	122·0	26017·4	18·7
Zichron	2186	21895·0	12008·0	4192·0	38095·0	18·9
Merchavia	1420	30166·0	2030·5	212·0	32408·5	22·8
Ein Harod	2425	20484·6	3389·0	233·0	24106·0	9·9
Yavniel	1385	21284·6	2344·0	327·0	23955·6	17·3
Kinereth	915	23534·6	6833·0	1157·0	31524·6	34·4
Rosh Pina	2349	22822·6	2791·7	592·0	26206·3	11·1
Total	21579	P 224582·4	47222·1	7712·5	269517·0	12·5
Average		P 24953·6	4135·8	856·9	29946·3	

\* Under this item are included also the salary and travelling expenses of the Senior Sub-inspectors.

Taking the results as a whole, it is evident that in seven of the nine demonstration areas there was a notable reduction in the malaria incidence. The percentage of reduction varied in the different areas, but it was sufficiently striking to indicate the possibility of malaria control. The average decrease for all the nine areas as compared with the incidence of 1922 was just short of 50 per cent.

*Cost of Control.* The question whether this measure of control can be obtained at a reasonably low cost is answered by the data given in the last two columns of summary in Table VIII. In those columns are given the total and *per capita* cost in each of the control areas. The total cost per Control Unit did not vary much for the different districts, since the principal item was the salary of the malaria inspectors. There is, however, a considerable variation in the *per capita* cost; it varied from a minimum of piastres P 5·0 (\$0·23) in Ecron to a maximum of piastres 34·0 (\$1·55) in Kinereth. This difference was to be expected, owing to the variation in the density of the population per Unit area. In Judea the density of the population is relatively high, while in the Valley of Esdraelon and Galilee it is very low. Since the total cost will remain the same or even decrease as the density

of the population increases, it is apparent that the *per capita* cost of the control work will diminish progressively, as the population grows. Even at present, however, the *per capita* cost compares favourably with that of the Antimalaria Demonstrations in the U.S.A. (6).

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## EXPLANATION OF PLATES VI-IX.

## PLATE VI.

Figs. 1-3. The plain after a heavy rain. The rush of water from the hills fills the dry stream beds, which are converted into rushing rivers, whose banks overflow, and flood the countryside. The rush of water is so powerful that it dislocates bridges and tears immense holes in the wadi beds.

## PLATE VII.

- Fig. 1. A cleaned pool in a winter wadi. When cleaned of algae and water weeds the pool is no longer favoured by *Anopheles elutus*.
- Fig. 2. Control of breeding by regulating a stream. This picture shows the same wadi as in Fig. 2, Pl. VIII, after it had been regulated.
- Fig. 3. Control of a large spring swamp by alternation of flow. Two large channels were cut from this spring, one north and the other east, and the water sent alternately five days in the north and five days in the east channel. The water is also used for irrigation which is thus automatically regulated.

## PLATE VIII.

- Fig. 1. A deep pool in a winter wadi, eroded by the flood and remaining the entire year. This picture shows a pool immediately after the rains. The rains have an excellent scouring effect.
- Fig. 2. A flowing spring stream, full of stones and overgrown with algae. The algae and pools under the stones offer excellent breeding places for *A. superpictus* and *A. sergenti*.
- Fig. 3. Pools similar to the one in Fig. 1, overgrown with algae, ranunculus and marsh grass, and breeding *A. elutus (maculipennis)*.

## PLATE IX.

- Fig. 1. Shepherds' watering holes—excellent breeding places for *A. elutus (maculipennis)*.
- Fig. 2. Another type of breeding place. A temporary pond formed in a blind pocket and remaining for two or three months after the rains; until June or July 1—long enough to be a serious cause of malaria.
- Fig. 3. Vegetation in overgrown wadis. In such places Paris Green is the most effective larvicide.

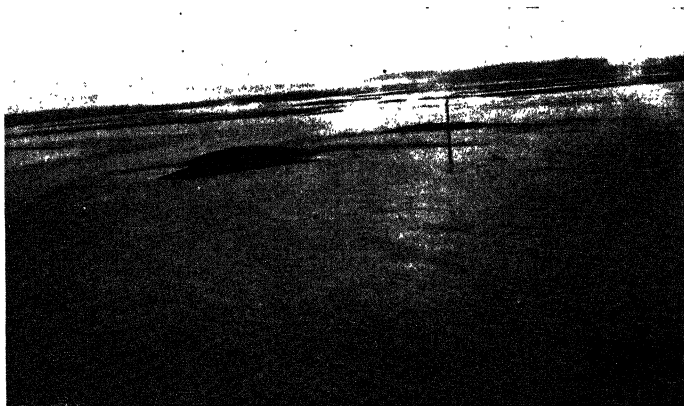


Fig. 1



Fig. 2



Fig. 3





Fig. 1



Fig. 2



Fig. 3



Fig. 1



Fig. 2



Fig. 3







Fig. 1



Fig 2



Fig. 3



# STIMULANTS TO BACTERIAL VARIATION.

By ARTHUR EASTWOOD, M.D.

(*From the Pathological Laboratory of the Ministry of Health.*)

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## INTRODUCTION.

IN a previous article on the capillary endothelium in relation to antibodies<sup>1</sup>, I suggested that the immune substances which are present in the living, actively immune animal may differ, both in their properties and in their range of action, from the antibodies demonstrable experimentally in an animal's serum, and that these differences may partly account for the greater efficacy of the former in the living body. I now wish to supplement the ideas which I there put forward.

There are many problems about the relations of parasitic bacteria to the animal body which cannot be satisfactorily explained by "antibodies," even if, as I proposed, this term be expanded so as to include new kinds and qualities of substances in addition to the properly authenticated serological antibodies. Various other factors have also to be considered. The one which I have made the subject of the present article is the influence of stimuli upon the vital capacities of bacteria.

When a cell grows in normal fashion, the "growth impulse" is taken for granted, as though it were simply part and parcel of the ordinary food supply. But growth may be accelerated, retarded, altered in character, or completely

<sup>1</sup> *Journ. Hyg.* xxii. p. 355, 1924.

inhibited without any evidence of deficiency in the quantity or quality of the material from which the cell obtains its appropriate "building-stones." Under such circumstances it is realised that something more is needed, something in the nature of a "stimulus," to start, regulate, and maintain the metabolic processes of hydrolysis and dehydration which are necessary for growth.

The reality of such stimuli is admitted, and it is recognised that they are equally of importance in accounting for normal growth and for deviations from the normal. They have not been isolated as chemical entities because the dynamics of living matter are too complicated. Still, they are chemical and physical agencies of one sort or another; they are not "vitalistic" in the sense of being something quite different from ordinary material forces.

Then what is a "stimulus"? The answer must be offered in a series of stages which involve the raising of other questions.

What are antigens, antibodies, and alexins? These are all highly respectable terms, the importance of which is taken for granted. But their definitions are vague and unsatisfactory. An antigen is something which causes the animal body to produce an antibody and it is then found that the antigen reacts specifically with this antibody; the definition of the latter is similarly expressed in terms of antigen; alexin is some property of fresh serum causing the completion, *in vitro*, of some reaction (generally of the antigen-antibody type) which would not be completed in its absence. One may elaborate these definitions with much detail, but one can do very little to clear up their obscurity; these postulated substances simply are what they do, and they have to be defined in terms of each other. Similarly with "stimuli." They are indispensable and highly respectable household words in all treatises on physiology and the kindred sciences, but it is often impossible to define them except in vague terms implying that they are the "something" to which an observed change is due.

Coming now to stimuli acting on the bacterial cell, how do these differ from other agencies which also act on the bacterium? In reply, one must first note that "other agencies" do not necessarily imply different and distinct substances, since a particular substance may initiate more than one kind of change in the cell. With this proviso, it may be said that a stimulus acts on the living cell and the result of the stimulus depends on the cell's own activities, any deviation from the normal (if such there be) being due to a change in the cell's metabolic processes; whereas other agencies, such as antibodies, enzymes, and non-specific toxins, act on some of the chemico-physical constituents of the cell, whether the cell be alive or dead, and produce results which are a direct consequence of this action and not a consequence of the cell's activities as a living organism. Thus substances which are antibodies or toxins are not, in these respects, cell stimulants, though they may also act as stimulants; *e.g.*, growth in immune serum may produce a bacterial variant and the presence of arsenic in a medium may induce the growth of a strain which is more resistant than the normal to this poison.

One naturally endeavours to form some sort of a mental picture which is a little more concrete than the bald statement that a stimulus is what it does.

When a bacterium is growing, there is a constant and systematic succession of changes in the loose attachment of chemical groups to the more complex and stable constituents of the bacterial protein; these changes, being associated with hydrolysis and synthesis (or dehydration), may be regarded as being, in one important aspect, changes in affinity for water on the part of the cell's component elements.

When a bacterium is in the resting stage but still alive, *i.e.*, capable of further growth, this succession of changes is not taking place.

But an alteration in the environment may convert the resting phase into the growing phase, *i.e.*, it may provide a growth stimulus, the explanation being that elements in the new environment produce, in the affinities of the bacterium for water, an unstable condition which leads to successive changes in a special order of sequence. This organised system of instability may be regarded as due to the normal growth stimulus.

But it is also possible for the environment to create a condition of instability which differs from the normal in some respect. Then the environment may act as a stimulant to bacterial variation, owing to a change in the attachment of particular chemical groups to the bacterium, and to a consequent change in the affinities of the bacterium for water.

This crude mental picture is an attempt to visualise and to reconcile the simplicity and the complexity of vital processes—simplicity as regards varying affinities for water, and complexity of the conditions determining these affinities.

#### TRANSMISSIBLE BACTERIAL AUTOLYSIS.

In a former report on bacterial variation and transmissible autolysis<sup>1</sup> I did not attempt to discuss the existence of "bacteriophage" as a living virus, since it appeared to me that the arguments against this view were so strong as to be practically conclusive. I therefore started with the assumption that "lytic principles" act as stimulants to bacterial cells but are not living organisms. I then proceeded to consider their mode of action. As the phenomena of transmissible autolysis are found only to occur with living and actually growing bacteria, I suggested that the lytic stimulus probably exerts its influence at the critical stage of cellular subdivision. My development of this view may be expressed briefly as follows.

It is desirable first to pay attention to the intimate way in which two essential processes of bacterial life are dependent on each other, *viz.*, the enzyme action which breaks up the food supply into particles suitable for assimilation and the synthesis of these particles to form living protoplasm. One feature of enzymes is that, after forming unstable combination with the material (substrate) undergoing digestion, they become dissociated from this

<sup>1</sup> Ministry of Health. *Reports on Public Health and Medical Subjects*, No. 18, 1923.

union and are left free to attack fresh substrate. But conditions may supervene, particularly in association with living matter, which tend to stabilise (instead of dissociating) the union between enzyme and digested food material, the result being that enzyme action ceases to be progressive and is replaced by a synthesis between substances formerly acting as enzymes and material upon which they acted.

Starting with this idea that the cellular enzymes of bacteria are not merely catalytic agents acting on a substrate, but are also the nucleus upon which bacterial protoplasm is built up, it would seem that, when a bacterium begins to grow, catalytic action upon its environment is at first predominant; then it passes through a critical phase which fixes or stabilises the constituents of bacterial individuality, a phase which is marked by the transition from catalytic action on the raw substrate to synthetic action on the elaborated product. When full development is followed by division, these processes are started afresh. Bacteria breed with that uniformity which is characteristic of species because both catalytic and synthetic action are attributes of the same protoplasmic substances.

Opportunities for variation (within the limits of species characteristics) are provided during the "critical phase" of transition from catalysis to synthesis, and the characters of the new generation will depend on the precise conditions which prevail when this phase is terminated by subdivision.

Subdivision may take place prematurely, before one or both of the segments have acquired sufficient stabilised protoplasm for independent growth. The result will be autolysis of those daughter-cells which lack this stability, and their dissolved products, by stimulating other bacteria to premature division and consequent autolysis, will act as a "lytic principle<sup>1</sup>."

It is of the greatest importance to note that lytic principles are not merely destructive agents causing the autolysis of bacteria; if they were, they would not be transmissible, since the survival of living and growing bacteria is necessary for their continued propagation. They are essentially stimulants to bacterial variation, giving rise to viable as well as to non-viable variants. Moreover, it has been shown, by experiments *in vitro* on successive generations of bacteria which have each been exposed to a lytic influence, that the surviving variants are not all alike. After the first action of the stimulant, some of them are (*a*) more resistant than the normal and some, though just able to survive, are (*b*) more sensitive than the normal. The process is repeated with the descendants of *a* and *b*; the weaker individuals die out and the stronger survive, perhaps including among the latter a few more hardy descendants of what were previously *b* forms. Carried to its conclusion, the process results in the production of a pure strain of highly resistant *a* forms, the other variant (*b*) having died out. Thereupon the pure *a* strain behaves like a normal culture, though more robust than the normal because the tendency to variation

<sup>1</sup> It is also possible that some lytic substance may escape from such cells before they arrive at the final stage of death from autolysis.

has been eliminated; it is no longer lysogenic, because the sensitive variants which, on disintegration, yielded the lytic substance, have disappeared. Perhaps the main interest of these experiments *in vitro* is that they raise the question whether similar stimuli may not be operative in the animal body, leading to the production of "resistant" and "sensitive" variants and thus helping to determine the animal's susceptibility or resistance to bacterial invasion. Is "epidemic virulence" evolved in this way?

The initial stimulus which produces transmissible lytic substance may be derived from many widely different sources, *e.g.*, faecal extracts from healthy or diseased persons or from healthy animals of various species, urine, diluted sewage, extracts of normal organs, peritoneal exudates of guinea-pigs inoculated with some bacterium, mixtures of bacterial culture and leucocytes, and so on. It may emerge "spontaneously" in an old culture; it has been obtained by treating bacteria with immune serum, by allowing the filtrate from a broth culture to act on fresh living culture, and by various other methods, such as shaking bacteria in distilled water or treatment with a small quantity of sublimate.

Hence it is extremely improbable that the stimulus is due to any one special substance. This is in accordance with the general fact that various physiological stimuli which produce the same effect are often of entirely different natures.

When a lytic principle has been obtained and is found to be transmissible, its precise range of action cannot be foretold; it is necessary to work it out by detailed experiment. For example, it may act only on the particular strain of the species from which it was derived, *e.g.*, a strain of *B. coli*, or it may attack the majority of strains of *B. coli* and also other species, such as dysentery and typhoid bacilli. The reason for these differences must, of course, depend on the special characters, chemical and colloidal, of the bacterial surface with which the lytic principle is brought into contact. But what these special characters are is unknown; one has simply to accept the fact that such differences exist. This, again, is in accordance with the general physiological experience that different cells may respond differently to the same stimulus.

#### OTHER FEATURES OF BACTERIAL VARIATION.

I do not think that transmissible autolysis is a phenomenon *sui generis*, but rather that it is to be associated with other manifestations of stimulation leading to bacterial variation.

This opinion is based on the following assumptions. (1) The influences which initiate and transmit autolytic change are not attributable to any sort of living viruses which prey on the bacteria, but are chemico-physical stimulants acting on the bacterial cell. (2) They are not simply "lytic principles"; besides causing growing bacteria to split off a number of non-viable forms which undergo autolysis, they affect the surviving daughter-cells in other ways.

(3) These modifications are not simply enhanced resistance or sensitiveness to a lytic influence but may affect other characters of bacterial growth. (4) As lytic changes may be initiated, in the same bacteria, by one or other of a great variety of causes, it is not likely that the original stimulus is some distinctive substance possessing chemico-physical properties specially associated with lytic action.

Apart from a tendency to autolysis, bacterial variants, whilst retaining the general characters of their species, may deviate from what is taken to be the normal in such respects as:—appearance of the colonies, morphology of individual bacteria, motility, enzyme action, capacity for growth, antigenic properties, and virulence.

What is likely to be the starting-point of such variations? In the life of an actively growing bacterium, the incidence of what I have termed the “critical phase” in the transition from catalysis to synthesis of food-material may be especially favourable for the disturbing action of a stimulus. When a young culture is growing under favourable conditions, without the production of any variants, it may be presumed that the stabilising influence which promotes synthesis acts exactly at the right time and in the right way. But, under other circumstances, a stimulus may act differently, causing the stabilising influence to operate (*a*) a little too soon, or (*b*) a little too late; and the consequences may be, respectively, (*a*) failure of some of the new generation of bacteria to develop their full biological properties, *e.g.*, loss of some function or of some antigenic constituent, or (*b*) the acquisition—after full development of biological potentialities—of some additional chemical groups which temporarily mask one or more of these potentialities, *e.g.*, disappearance and subsequent return of some biological property. And one might amplify these possible causes of variation by imagining that the intervening stimulus caused the stabilising influence to behave “not quite in the right way,” thus producing some deviation from the normal molecular or colloidal arrangements for the synthesis of chemical groups into protein. Under this general view, autolysis is comprised as simply an extreme instance of variations in the daughter-cells, the disorganisation in the cellular mechanism being so great as to be incompatible with continued existence as an organised cell.

In relation to the causes of bacterial variation, probably much significance is to be attached to the fact that they commonly arise “spontaneously,” *i.e.*, they arise *in vitro* without the introduction of any extraneous factor into the culture medium. This is frequently observed to be the case whether the variation be slight or profound. It may consist of no more than a slight irregularity in the synthesis of bacterial protoplasm, characterised by one feature only—irregularity in the disappearance and reappearance of a particular antigenic component; a condition, in fact, where it may be difficult or impossible to say which is really the “normal” bacterium and which is the “variant.” Further changes supervene when there is opportunity for more prolonged action of the “spontaneous” modifying influences which may be



present *in vitro*. The colonies formed by the variants are distinctive; there is more profound alteration in agglutinability and agglutinogenic capacity; there is less tendency of the variants to revert to the parent form; then the variants, though still viable *in vitro*, are found to be non-viable *in vivo*, *i.e.*, they have lost their virulence; finally, the variant fails to survive even *in vitro* and succumbs to autolysis.

These facts suggest a comparison with the influences which are liable to produce bacterial variants *in vivo*. As the bacterium is placed in an entirely different environment, the influences now operative may be considered to be "extraneous" rather than "spontaneous." Still, when one considers results, there is a broad and very striking resemblance—though not a resemblance in every detail—between variants (including non-viable forms) produced *in vivo* and those produced "spontaneously" *in vitro*. This is not particularly surprising, since different agents, acting as physiological stimuli, may produce identical effects. It does, however, suggest an important point, *viz.*, that the new (animal) environment does not impose on the bacterium entirely new influences of a "foreign" or drastic nature but rather induces the bacterium to "modify itself" by employing very much the same mechanism which was operative in the test-tube.

It is quite probable that, alike in the animal body and in the test-tube, the commonest and most important cause of bacterial variation is neither an alteration in the food supply nor a change in the nature of the bacterial enzymes but some slight change in the environment which modifies the bacterial surface as regards its affinity for water.

#### THE INFLUENCE OF SYMBIOSIS.

##### *Symbiosis amongst bacteria.*

Bacterial growth may be initiated from a single cell, and the culture so obtained may, under suitable conditions, be propagated indefinitely; this is naturally to be expected with unicellular organisms.

But there is considerable evidence that the individual cells are not absolutely independent entities. Bacteria often help each other to grow. When a medium is seeded with a particular strain, growth may fail when the amount of inoculum is very small but succeed when it is larger; or, if the smaller seeding does not simply die out, rapidity and final abundance of growth may be greater when more bacteria are transplanted.

This evidence of co-operation between individuals of the same strain seems best explained by the assumption that each cell forms temporary union, by adsorption on its surface, with some substance secreted by the adjacent cells (or, in some cases, with the autolysate of such cells), and that this substance increases the cell's capacity for assimilating food and accelerates the events leading to its subdivision.

After a time, growth becomes less rapid and finally ceases. These changes

may mean that the food supply is becoming used up or is rendered useless by the accumulation of inhibitory waste products; but there is another influence which must also be considered. The continued adsorption of cellular products may have reached a stage of equilibrium, the successive phases being (1) a stimulus to metabolism and reproduction caused by these products, (2) a slowing down and, finally, (3) an inhibition of these processes, when the cellular products have been adsorbed in excess of the optimum required for stimulation. The suggestion, therefore, is that bacterial multiplication may, to some partial extent, be controlled by a self-regulatory mechanism, irrespective of changes in the nutritive characters of the medium.

If the bacteria in an undoubtedly pure culture (derived from a single cell or a single colony) were all exactly alike, it would suffice to form a quantitative conception of this influence of bacterial secretion upon bacterial growth, amounts increasing up to a maximum,  $x$ , being stimulative, and amounts exceeding  $x$  being unfavourable to growth. But it is now known that individuals in a pure culture may differ from each other; therefore the qualitative character of this influence has also to be considered.

Changes found on plating out a pure culture, particularly if it is old, may relate to the type of colony, antigenic properties, virulence, viability, or to other characteristics. Though such changes cannot be attributed to one factor alone, the influence of bacterial secretions or disintegration products, this factor cannot, as a rule, be excluded. For example, if some of the variants show the phenomenon of transmissible autolysis, it may be taken that dissolved bacterial substances were the starting-point of this change.

Similar considerations apply to mixed cultures. Species B may promote or retard the growth of species A. The action may be partly indirect, *e.g.*, B may convert the culture medium into one which is more favourable or less favourable for the growth of A; but direct action must also be included as part of the process, *viz.*, modification of A by the adsorption of substances derived from B. And the effect, as before, may be partly quantitative—on the output of new growth, and partly qualitative—on the characters of the new cells.

Leaving aside for the moment the question of distinction between a "stimulant" and a "building-stone," it may be noted that, when bacteria are growing together in pure culture, variants probably arise because the combining affinities of the surface of the bacterial cell for the materials in its environment are not absolutely fixed and immutable. For example, an affinity may be satisfied, or partially satisfied, by union with any one of three different kinds of material,  $a$ ,  $b$  and  $c$ , which may all be present in the environment. It may be a matter of chance whether union is actually formed with  $a$ , or  $b$ , or  $c$ ; but a bacterium which has united with  $a$  may be slightly different from one united with  $b$ , or  $c$ , and such differences may make a difference in the nature of the subsequent affinities of the bacterium and in the character of the materials which unite with it.

Or, again, in this "rivalry" between  $a$  and  $b$ , much will depend on the rate of combination; if it is the same for each, there will be a tendency for

the number of bacteria affected by the  $a$  influence to be about equal to the number affected by  $b$ , and this may be the starting-point for the differentiation of a culture into two varieties (A and B) each in about equal numbers; if  $a$  or  $b$  has the more rapid rate of combination, there will be a tendency for A or B to preponderate.

And there may be another important difference between  $a$  and  $b$ ; the former may make a much firmer union than the latter with the bacterial surface. Here, again, the ultimate consequence may be some structural difference between the A and the B forms, perhaps a difference in the firmness of the synthetic combinations of assimilated food-material. Hence, perhaps, the one variety may be more resistant to hydrolysis than the other—a type of resistance which may be an attribute of virulence.

In discussing symbiosis as a factor in the production of variants, it must be remembered that this is only a one-sided view. Symbiosis is probably of equal importance in the repression of variants. It is now admitted that individual bacteria in a pure culture are never all exactly alike; and the statement no longer causes any surprise. One is rather inclined to wonder why such differences do not cause more confusion, why, for example, it is possible to obtain practical identity of all important biological properties by growing two subcultures of the same strain under identical conditions. The reason seems to be that, under appropriate conditions and with young and actively growing cultures, symbiosis tends to encourage the propagation of the dominant characteristics and to inhibit the development of the weaker variants.

#### *Symbiosis between bacteria and the animal body.*

Enquiries into bacterial infections are generally based on the assumption of antagonism between the bacterium and the animal host. Quite rightly—up to a certain point. But there is a danger of laying too much stress on this pictorial idea of “warfare,” of a constant battle, ending in the survival of the fittest, between the parasite and the invaded body. It must not be forgotten that the animal body may not always be aware that bacteria should be treated as “enemies” but may, on the contrary, actually facilitate their growth.

In relation to bacterial growth the animal body may be considered as the source of two distinct kinds of material:—(a) stimuli (favourable or unfavourable) to such growth and (b) the supply of food as raw material which is convertible into bacterial protein.

The animal's stimulus to growth may be regarded as particularly favourable in the case of certain strictly parasitic micro-organisms. The leprosy bacillus, for example, seems unable to grow without the stimulus of something derived from the living cells of certain species of animals, something which there is no reason to postulate as being a special constituent of the protoplasm of the leprosy bacillus. Perhaps a similar animal stimulus is required for the growth of some of the invisible viruses which have not been cultivated *in vitro*.

Moreover, many of the ordinary parasitic bacteria which are capable of survival and growth in the saprophytic condition (their own bacterial secretions being a sufficient stimulus) often grow better in the body of a susceptible animal (*i.e.*, when aided by the stimulus derived from animal cells), *e.g.*, plague and anthrax bacilli and other organisms which produce an intense bacteriaemia in their host. Here, again, there seems to be a distinction between the bacterium's own growth impulse and the stimulus to growth which is derived from the host.

A distinction between the two kinds of stimuli may also be observed in cases where the one derived from the animal is relatively unfavourable to the bacteria. The tissues of cattle, for example, are relatively unfavourable to the growth of the "human" type of tubercle bacillus; but this inhibitory action may be overcome by artificial means, *e.g.*, by intravenous inoculation of large doses of the bacilli; and it is known that attempts to immunise cattle with living "human" tubercle bacilli are unsafe, since they may survive and multiply for lengthy periods and may be excreted in the milk. Similarly it has been shown experimentally that avian tubercle bacilli can be made to grow profusely in mammals. In these exceptional cases where natural resistance has been overcome, the bacteria have been able to supply their own stimulus to growth in excess of the adverse stimulus attributable to the fluids of the animal body.

But it is not always the case that the body fluids of a particular animal consistently act on the same type of bacterium in the same way; they may be favourable to its growth on one occasion and unfavourable on another. For example, a small dose of culture of tubercle bacilli is inoculated into a guinea-pig and produces a local lesion which leads to slowly progressive and eventually fatal disease; when the disease is established but before its termination, a small dose of the same culture is inoculated into a site of the body which has not yet become infected; this second site is found to be resistant and a tuberculous local lesion is not produced. Why this difference between the effects of the first and the second dose? As the bacilli were identical to begin with, it must be due to some difference in the action of the body fluids on the bacteria. On the occasion of the first inoculation, these fluids enabled the bacilli to acquire and retain invasive capacities; on the occasion of the second, the body fluids prevented the acquisition of invasive capacities though not interfering with the retention and transmission of such capacities which had already been acquired.

The feature of special importance is that this change in the properties of the animal fluids is the result of infection.

#### COMPARISON OF BACTERIAL WITH ANIMAL CELLS.

At this point it is interesting to consider possible resemblances between bacterial and animal cells in respect of some of the conditions which influence their growth.

(1) As regards symbiosis there is, of course, no comparison between the elaborate regulatory mechanism of the animal body and the growth of bacteria. But a suitable parallel may be found in the cultivation of tissues *in vitro*. Here it has often been noticed that growth takes place when the tissue cells are numerous and in close proximity to each other but fails in cells which are isolated.

(2) What causes the embryonic cell to change into the adult type? It has been suggested in the case of bacteria that adsorption of more than the optimum amount of bacterial secretion may help to account for the slowing down of bacterial growth; possibly, when embryonic cells have accumulated up to a certain amount, there is a similar excess of cellular secretion, which now has a retarding effect on growth and leads to some qualitative change in the new generations of cells. Observations on tissue cultures in a medium containing plasma derived from animals of different ages apparently indicate that, after the embryonic stage is terminated, the plasma may have a restraining influence on growth in the living body, and that this influence increases with the age of the animal.

(3) During the processes of recovery after trauma and in inflammatory reactions, there is again reversion to a more actively growing, though not actually embryonic, type of cell. Here the new cellular stimulus is probably attributable, at least in part, to adsorption of material from disintegrated cells, since it is known experimentally that growth may be stimulated by extracts of leucocytes or of various types of tissue cells. Recovery is accompanied by reversion to the normal adult type. These processes may present some analogy to the changes produced in a bacterial culture which, after becoming stabilised on artificial media, is transferred to a new environment, such as the body of a susceptible animal, which provides fresh stimuli to the bacterial cells. The effect may be a reversion to some of the characters which have been temporarily lost on the artificial medium; on return to the old environment, these characters may again disappear.

(4) Is there, as some authors have suggested, anything comparable between transmissible bacterial autolysis and the autolysis which is a constant feature of malignant growths? There are certainly very obvious differences. With bacteria the two most striking features are that the lytic principle (1) is usually propagated through successive cultures of normal cells, and (2) makes normal cells become abnormal, *i.e.*, lysogenic. In malignant disease, (1) autolysis is transmitted through abnormal (malignant) cells; and (2) the lytic principle does not convert normal into abnormal cells; it may stimulate the growth of normal adult tissue *in vitro*, but it does not cause such tissue to assume the malignant type. Still, it is just possible to find a parallel with respect to one feature which is observed as the result of continued exposure of bacteria to a lytic agent through many generations. I do not refer to the end result, which, in a successful experiment, is the production of a pure culture insusceptible to lytic action and non-lysogenic; I am thinking of that inter-

mediate stage where the bacteria are more resistant than the normal to lytic action but are still partially susceptible to lysis and still retain lysogenic power. This stage may present some resemblance to what one may imagine to be the development and propagation of the malignant cell. Owing to some stimulant which is slightly different from the normal, a normal cell divides into two daughter-cells, of which one (*a*) is normal and the other (*b*) non-viable. It may accidentally happen that the autolysed remains of *b* stimulate *a* to divide into *a*<sup>1</sup> (apparently normal and viable) and *b*<sup>1</sup> (non-viable). A similar action may be repeated in succession by *b*<sup>1</sup>, *b*<sup>2</sup>, *b*<sup>3</sup> etc. on the viable descendants of *a*<sup>1</sup>. This chapter of accidents may come to an end without anything further happening; or it may terminate because the descendants of *a*<sup>1</sup> become more sensitive than the normal to lytic action and all fail to survive. Another possibility is that some of these descendants become more than normally resistant to lysis, though still partially susceptible and still lysogenic. Such cells would possess features specially associated with malignancy; in virtue of their lytic principle, they would exert and transmit to their offspring a powerful stimulus to premature proliferation and, in virtue of their relatively high degree of resistance to autolysis, the cells which survived would exceed, from generation to generation, the number which perished. Thus there would be a similarity between this (the final) phase of transmissible autolysis amongst malignant cells and that intermediate stage of the same process amongst bacteria to which I have called attention. The resemblance is masked because there is usually a marked difference in the end results. With bacteria, in the ordinary test for lytic action, autolysis generally takes place very rapidly and the lytic phenomena are then more conspicuous than the stimulus to new growth; with malignant cells in the animal body, autolysis is slower and cellular proliferation is the predominant feature.

(5) When either bacteria or animal cells have acquired virulence, *i.e.*, capacity for invading the tissues of the animal body, it is found in both cases that this capacity is inherent in the cells themselves and, given a favourable environment, may be transmitted *ad infinitum* to successive generations of cells. The interesting point is that, in order to retain this capacity, the bacteria must remain normal, *i.e.*, must be fully equipped with their ordinary mechanism for metabolism, whereas the animal cells must remain abnormal, *i.e.*, must retain a degenerate form of metabolism characterised by imperfect assimilation of food and premature subdivision.

(6) The same stimulus may be favourable to one kind of cell (promoting vigorous growth) and unfavourable to another (causing degeneration and autolysis). Thus the stimulant action of the animal body helps one species of bacterium to grow (natural susceptibility) and inhibits the growth of another (natural immunity). Similarly, treatment of an animal by various means (*e.g.*, inoculation of defibrinated blood or olive oil or application of *x* rays) may cause the animal to produce a stimulus which increases the growth of some cells (*e.g.*, lymphocytes) and inhibits others (*e.g.*, a cancer).

graft). It is to be noted that an overdose of the agent, such as  $x$  rays or olive oil, does not cause the animal to produce a stimulus but is merely noxious and lowers the animal's general powers of resistance.

(7) For the distinction between (*a*) stimuli to bacterial growth and (*b*) material utilised by the bacteria as food a parallel may perhaps be found in observations on the cultivation of animal tissues *in vitro*. Drew, for example<sup>1</sup>, has shown that for the growth of adult tissue two different substances are requisite, (*a*) one which acts as a stimulus and (*b*) one which furnishes the nutritive material. Extract of embryonic tissue acts as (*b*) and he has not been able to find any substitute for this. When the cells are implanted on the ordinary medium containing (*b*), there is a lag of many days before growth commences. During this period of delay some of the implanted cells undergo autolysis and it is the accumulation of this autolysate which acts as (*a*) the stimulus to growth. The lag may be abolished by planting the cells on a medium which already contains (*a*) in the form of an artificially prepared autolysate of adult tissue. When growth has started, it may be continued indefinitely by transplanting to media containing (*b*) alone; if, however, the transplants are made on media to which artificially prepared (*a*) has also been added, "early degeneration with ultimate death of the whole culture takes place." Thus there is a sharp distinction between the nutritive properties of (*b*) and the stimulative properties of (*a*); and the stimulus of (*a*) may be either favourable to the initiation of growth or, if cumulative, unfavourable to continued propagation.

#### IMMUNITY.

##### *Natural immunity and natural susceptibility.*

To begin with stimuli associated with natural immunity. It is reasonable to suppose that the property which confers immunity is associated—though not identical—with the property conferring those species characteristics which are demonstrable, serologically, by the precipitin test. One must, I take it, explain the individuality of an animal's serum by assuming that the different cells of the body split off certain by-products which find their way into the plasma and there form certain chemical and colloidal combinations; these combinations, though unstable and constantly changing *in vivo*, are characteristic of the species and become stabilised in the serum in certain characteristic ways. In the naturally immune animal some of these circulating substances are chemically equipped in such a way that they (1) are adsorbed by the bacterial surface and, thereupon (2) split off, or otherwise interfere with, components which are linked to the bacterial protoplasm and are necessary for its vitality, *i.e.*, they prevent the progress of the dehydration synthesis or, possibly, combine with a bacterial enzyme in such a way as to render it inert.

But the association, amongst animals, between natural immunity towards

<sup>1</sup> *Lancet*, I. p. 833, 1923.

particular bacteria and serological blood-relationship does not amount to a strict correlation of the two properties. Closely related animal species sometimes differ sharply in immunological respects. Moreover, the combining properties which confer natural immunity in the circulating plasma are generally labile and disappear in the serum, whilst the serum, though not antibacterial, retains its species characteristics. Hence it has to be admitted that these special combining properties seem to be a more or less accidental result of the animal's biological individuality.

Similarly, natural susceptibility can only be described, in the present imperfect state of knowledge, as being "accidental"; it means that those elements circulating in the plasma which are the expression of the animal's biological individuality do not possess the same sort of combining affinities for substances attached to the bacterial protoplasm; and no explanation for this circumstance is available.

It may seem disconcerting to confess that, in the present lack of knowledge, the property which makes one animal naturally immune and another naturally susceptible to a particular bacterium is simply an "accident" or "incident" which cannot be explained. Recognition of the difficulty leads, however, to some reflections as to the nature of the action exercised by the immune or the susceptible animal on the bacterium. The very fact that the character of the influence, in relation to different animal species, appears so irregular and haphazard suggests an important group of physiological actions in which this lack of demonstrable correlation between cause and effect is a conspicuous feature. I refer to the phenomena of cellular stimulation and cellular inhibition.

Perhaps I ought to elaborate this point a little more. There is no evidence that the naturally immune animal does not possess a practically unlimited supply of food material which is suitable for the nutrition of every known parasitic bacterium; in this respect there is no difference between the immune and the susceptible animal (*e.g.*, the difference between the fowl's and the rabbit's susceptibility to anthrax is not due to differences between fowl protein and rabbit protein). It has not been found possible, either by chemical or biological analysis, to obtain from the naturally immune animal some substance which has a specifically toxic or lytic action on the bacterium towards which it is immune; in fact the search for such specific substances has been abandoned as hopeless.

Still, there must be some unknown cause which would account for the difference between natural immunity and susceptibility. It seems to me that it probably depends on the extreme sensibility of the bacterial surface to very minute differences in the composition of the animal's plasma. These differences, I have suggested, are comparable to differences in physiological stimuli, being favourable to the cell's metabolism in one instance and inhibitory in another. And for the word "stimulus" one might substitute the rather more definite conception of an alteration in the cell's permeability, the idea being



that the immune animal modifies the selective ingress and egress of fluids in some way which is incompatible with the cell's internal processes of hydrolysis and dehydration, while the susceptible animal does not cause this interference in the normal permeability but may make the conditions of permeability actually more favourable to bacterial growth.

An unfavourable stimulus, as distinct from a toxin, may be thought of as a stimulus to continued catalytic action, unaccompanied by synthesis and resulting in bacterial death from autolysis. This may explain death of the anthrax bacillus, for example, in a naturally immune animal. Under exceptional circumstances, where the bacillus is excluded from the free action of the body fluids of such an animal, this stimulus is not operative and the bacilli may multiply, a fact which is additional evidence that inhibition of growth is not due to lack of suitable nutritive material.

The above considerations suggest that the problems of natural immunity and natural susceptibility do not necessarily await solution in terms of antigens and antibodies.

*Acquired active immunity.*

I propose first to consider certain features of immunity to the anthrax bacillus.

It is an old idea, and was suggested by Preisz in his work<sup>1</sup> on anthrax, that acquired immunity is in some way a reinforcement of natural immunity and that all animals, from the naturally immune to the most susceptible, possess some degree of natural resistance.

This view may be elaborated in various ways. To develop it in line with my suggestions in the preceding section, one would assume that in the circulation of the naturally immune animal there are substances which have combining affinities for the surface of the anthrax bacillus and alter its relations to its environment in such a way as to render it non-viable. These substances are highly labile; they disappear from the animal's serum and cannot be recovered from it; they cannot be "reactivated" by introducing the serum into the body of a susceptible animal.

The normal elements circulating in the plasma of a susceptible animal do not possess combining affinities which are strong enough to make firm union with the surface of the anthrax bacillus. When such an animal is actively immunised, the disintegrated protoplasm of the anthrax bacillus interacts with these normal elements circulating in the plasma in such a way as to increase their affinities for the surface of the anthrax bacillus. Perhaps, as I suggested previously<sup>2</sup>, the interaction to which this modification of the plasma is due takes place at the surface of capillary endothelium, and is brought about by filtration of the plasma through endothelial cells which have adsorbed disintegrated bacterial protein.

<sup>1</sup> This work is worth consulting in its original form (*Centralbl. f. Bakt. Orig.* XLIX. p. 341, 1909), as it contains many interesting and valuable observations.

<sup>2</sup> *Journ. Hyg.* XXII. p. 355, 1924.

In the first stage of active immunity, these modified elements of the animal body are, as in natural immunity, highly labile and disappear from the serum; the animal acquires immunity, though the serum has no antibacterial properties. Substantial active immunity may be established without any advance beyond this stage.

Attention has often been called to this fact, the production of active immunity to anthrax without the presence of demonstrable antibodies in the animal's serum. When discussing antibodies, I suggested<sup>1</sup> that phenomena of this nature may perhaps be explained by the existence in the living body of effective antibodies which, owing to their labile nature, disappear from the serum. In the present connection I wish to call attention to another aspect of this kind of immunity. What is the mode of action of these labile substances *in vivo*?

There is a difference between (1) disintegrated bacterial protein and (2) the surface of the living bacterial cell. In active immunisation, the change produced in the body is due to the stimulus of (1), but the new substances which are produced act primarily on (2). Their reaction with (2) may be (a) simply a union with the bacterial protein, analogous to the first stage of an agglutination reaction *in vitro*, or it may be (b) a modification in the chemico-physical condition of (2), due to loose contact followed by prompt dissociation and therefore in the nature of a stimulus which is not associated with the agglutinin type of union; and, theoretically, the reaction might be (c) a relatively firm union of the agglutinin type accompanied by a chemico-physical modification. The main point of practical interest is that *b* may occur without *a* (antibacterial action without agglutinins), just as *a* may occur without *b* (adsorption of agglutinin not incompatible with the vital properties of the bacterium). In this connection, it seems to me, the probable existence of this *b* type of reaction is more important than the question whether it is due to something which, if it could be obtained *in vitro*, ought to be called an "antibody."

It does not follow that active immunity in other bacterial infections is due to the same principles as in anthrax. In other cases, serological antibodies may be demonstrable and may be of considerable importance in the mechanism of such active immunity; still, they do not suffice to explain it completely and here, also, one of the factors may be a stimulant action on the bacterial surface as distinct from an adsorption of antibody by bacterial protein.

#### *Passive immunity.*

To start again with anthrax, if an animal which is already actively immunised receives repeated large doses of anthrax bacilli, fresh substances are found in the circulation. These, it may be assumed, are due to interaction between disintegrated bacterial protein and elements of animal protein. They

<sup>1</sup> *Journ. Hyg.* xxii, p. 355, 1924.

do not persist indefinitely *in vivo*, but, if the animal is bled before they have disappeared, they form highly stable constituents of the serum. When such serum is introduced into a susceptible animal infected with anthrax, it confers passive immunity; as it is not antibacterial *in vitro*, it is inferred that it probably acts *in vivo* not by direct combination with the bacilli but by "activating" the animal's plasma, *i.e.*, by breaking up some constituents of the plasma in a new way, so that they acquire new affinities for the bacterial surface, the result being that the animal's "growth stimulus," originally favourable for the anthrax bacillus, has now become unfavourable. This idea of the mechanism of passive immunisation in anthrax infections may be of importance.

The problems of anthrax immunisation have always been of special attraction to bacteriologists, because it is thought that their solution would throw light on general principles of immunology. They have not yet been solved. What I have written above is only one way of attempting to explain some of the difficulties or, more correctly, merely an attempt to restate an unsolved problem in terms which may, in some degree, approximate to its real nature. Is it reasonable to suppose that in other infections, as well as in anthrax, the same kind of mechanism is operative in natural, active, and passive immunity?

In the case of anthrax, the idea that both active and passive immunity are simply a reinforcement of natural resistance (already inherent in the susceptible animal) has generally been arrived at by the method of exclusion; it has not been possible to account for the facts by any sort of recognisable antibody to the bacilli and therefore it has seemed necessary to postulate indirect action, *viz.*, action of something (? antibody) on the tissues or fluids of the body, in consequence of which the latter behave antagonistically towards the anthrax bacillus. I have attempted to regard this inherent property of the animal body not as inherent "resistance" but as a cellular stimulant which may vary in its action on the bacillus, being sometimes favourable to its growth, sometimes (perhaps) indifferent to it, and sometimes adverse; then the acquired immunity (active or passive) of a susceptible animal would mean the change of its stimulant action from one which was favourable (or indifferent) to one which is adverse.

Would it be justifiable to attempt a similar explanation in the case of other bacteria? Not when acquired immunity can be explained in other and simpler ways, as by the direct action of a demonstrable antibody on the bacterium. Unfortunately, this cannot always be done, sometimes because there is active immunity without known antibodies, and sometimes because antibodies, though demonstrable, will not confer passive immunity. In such cases it may not be inappropriate to raise questions as to the significance of cellular stimuli.

## CELL STIMULI IN RELATION TO OTHER FACTORS.

*Leucocytic enzymes.*

It has long been recognised that phagocytic cells, particularly leucocytes, may protect the animal body against invading bacteria in other ways than by ingesting them. Leucocytes contain digestive enzymes, which are released when the cells undergo death and autolysis; and these liberated substances form part of the non-specific defensive mechanism in both natural and acquired immunity.

I may refer briefly to some of the older work on this subject.

Pettersson has been an important member of the school which regarded the leucocytes as a source of bacteriolysins. These substances, according to Pettersson<sup>1</sup>, were distinct from the serum bacteriolysins and differed from the latter in being more highly resistant to heat. Though they might act within the leucocyte after phagocytosis had occurred, they were not secreted from the living, uninjured leucocyte; hence he called them endolysins. He obtained them as extracts by repeated freezing and thawing of the leucocytes, and found that the leucocytes of different animals differed in their amounts of soluble bactericidal substances for particular bacteria.

Extracts from the leucocytes of an immunised animal were not very markedly more bactericidal than extracts from the leucocytes of a normal animal of the same species. This he considered natural, because leucocytes did not live for long and at the end of an animal's immunisation they were not likely to be very different in composition from what they were at the beginning.

The action of his leucocytic extracts was somewhat slow. He noted this particularly with pneumococci. At the end of 6 hours' incubation with the extract there was often a definite increase in the number of cocci; but this was followed by a rapid decline, which was well marked when plates were made 18 hours after the commencement of incubation.

In the course of further experiments, Pettersson also found some evidence *in vivo* that the injection of fresh leucocytes might afford some amount of protection against bacterial infection. The action of leucocytic extracts was weaker and more uncertain; but the addition of such extract to fresh leucocytes increased the protective action of the latter.

Important differences have frequently been noted between *in vitro* and *in vivo* experiments with leucocytes and leucocytic extracts. Weil and Nunokawa, for example<sup>2</sup>, produced large accumulations of leucocytes in guinea-pigs by intraperitoneal inoculation with broth; 18 hours after this injection, they introduced into the peritoneal cavity a small number of anthrax bacilli (in some cases not more than 200) obtained from the exudate of an anthrax-infected guinea-pig. Since rapidly fatal infection ensued, the leucocytes were evidently unable to exercise any protection. On the other hand, they found that guinea-pigs' leucocytes contained substances which were markedly bactericidal *in vitro*, not merely against bacilli from cultures or against strains known to be avirulent but, in an equal degree, against the capsulated forms of highly virulent strains taken fresh from the body of an infected animal.

In order to exclude the possibility of phagocytosis or the vital secretion of bactericidal material, they used in their first experiments the extract and deposit of leucocytes which had been killed by alternate freezing and thawing twice repeated. Not only was this material potent against virulent strains, but no increase of resistance could be developed

<sup>1</sup> *Centralbl. f. Bakt. Orig.* XLII, p. 56 (1906) and XLV, pp. 160 and 235 (1908).

<sup>2</sup> *Centralbl. f. Bakt. Orig.* LIV, p. 262, 1910.

in such strains by repeated passage through susceptible animals (guinea-pigs or mice). They then compared these results with the action, *in vitro*, of living leucocytes. Here again they found a definite bactericidal action against virulent, encapsuled anthrax bacilli, although in half out of the eight experiments recorded the action of the frozen leucocytes was more or less definitely stronger.

As there was no phagocytosis, the nature of this bactericidal action remained to be explained. They made three preparations: (1) their usual suspension of living leucocytes in serum; a similar suspension was allowed to stand for  $3\frac{1}{2}$  hours; it was then centrifuged and (2) the supernatant fluid was used; (3) the deposit of leucocytes was also used, after the addition of fresh serum. Eight samples of virulent "animal" bacilli were tested and uniform results were obtained. (1) and (3) were equally, and strongly, bactericidal; (2) showed little or no evidence of bactericidal action. Evidently, therefore, the leucocytes did not spontaneously excrete bactericidal substance into the surrounding fluid.

The authors concluded that anthrax bacilli, as they had previously found to be the case with the bacilli of swine erysipelas, had an affinity for the bactericidal substance within the leucocytes and removed it from these cells; then the bacilli were killed by "aphagocytic action."

This affinity was a property of several varieties of bacteria. They showed that killed bacteria of three sorts, typhoid, cholera, and "animalised" anthrax, on incubation with leucocytic extract for 1 hour at  $37^{\circ}$  C., were equally efficacious in destroying the capacity of the extract to kill anthrax bacilli. In this property of the bacteria there was no evidence of specificity; at all events bactericidal activity for a particular bacillus could be removed in a non-specific way. They also obtained evidence that dead bacteria had a similar, non-specific property of inhibiting the bactericidal action of living leucocytes.

In dealing with the bactericidal properties of leucocytic extracts in relation to their aggressin theory, Bail and Weil<sup>1</sup> observed that such extracts were highly destructive for "animal" anthrax bacilli, even more so than for culture bacilli. Moreover, they found that, when leucocytes were suspended in pure aggressin and frozen, this extract was highly bactericidal for animal bacilli; in fact, when the leucocytic endolysins (Pettersson) were once set free, the strongest concentration of an antibacteriolytic exudate could not inhibit their action.

They regarded the aggressin as an abnormal secretion elaborated by the anthrax bacillus during its growth in the animal body and not produced during growth under ordinary saprophytic conditions. This aggressin acted directly on the leucocytes, not by fixing bactericidal material given off by the cells but by preventing the leucocytes from releasing this material. Thus, when leucocytes were treated with anthrax aggressin and then centrifuged and washed, they were less bactericidal than normal leucocytes or than leucocytes treated with an exudate not produced by anthrax bacilli. But when the cells which had been treated with anthrax exudate were frozen and thawed, it was found that the bactericidal property of the extract was equal to that obtained from normal cells. The paralysis of leucocytes by aggressin was specific, *e.g.*, cells treated by anthrax aggressin were still active for *B. subtilis*.

I think the above extracts, taken from a very voluminous literature, will suffice to show that the older writers attached a high degree of importance to the direct lytic action of leucocytic enzymes upon bacterial protein. The subject possesses an equal degree of importance at the present day, notwithstanding the changes which have taken place in ideas about immunological reactions.

To correlate the older ideas with the subject of the present article, one

<sup>1</sup> *Arch. f. Hyg.* LXXXIII. p. 218, 1911.

must draw a distinction between (1) the action of leucocytic material as a stimulus to the vital properties of a cell (*e.g.*, properties concerned with capacity to perform those dehydration syntheses which are necessary for growth) and (2) its action as an enzyme upon the properties which living and dead protein possess in common. The older work was occupied with (2), though one may perhaps discover, in the light of recent knowledge, that it contains some links connecting it with (1).

The latent period in the action of Pettersson's leucocytic "endolysin" may possibly be attributable to the time required for the leucocytic extract to develop its properties as a stimulus to bacterial autolysis; and the partial protection which he found was afforded by the inoculation of leucocytes may also be associated with a "lytic stimulus."

The work of Weil and Nunokawa may be taken as indicating that the material which acts as a stimulus to the formation of lytic substance may be adsorbed by dead bacteria in a non-specific manner.

The attempt of Bail and Weil to reconcile their aggressin theory with the bactericidal properties of leucocytic extracts seems to me to be confusing and unsuccessful; their observations on the specific action of "aggressin" upon leucocytes may perhaps mean that the bacterial extract, present in their "aggressin," adsorbed lytic substances in a selective manner.

These are a few of the examples in which it may be found that the older writers called attention, perhaps unconsciously, to the importance of the fact that, when the body is invaded by bacteria, it has to deal with living protoplasm and not merely with foreign protein.

Interest in leucocytes as a source of cell stimuli is not confined to bacteriologists. One learns from recent work on the cultivation of tissues *in vitro* that, under certain experimental conditions, material derived from leucocytes appears to stimulate growth, and it also appears probable that such material is important, *in vivo*, as a stimulant to growth in processes of inflammation and the repair of tissues.

Perhaps these findings may be compared with the capacity of leucocytes to initiate transmissible bacterial autolysis, a capacity which, in my view, is in the nature of a bacterial stimulant. In this connection Bordet's opinion is worth quoting. Referring to his observations on intestinal bacteria and the phenomena of autolysis, he says<sup>1</sup>:—"External influences such as that of a leucocytic exudate modify the bacterium, inducing the latter to elaborate a lytic substance capable of diffusing itself and bringing about the same autolytic phenomenon through successive cultures."

It is thus possible that leucocytic material is equally a stimulus to cellular division with bacteria as with tissue cells, the difference being that, in the latter case, the new cells are presumably as normal as their ancestors, whereas, in the former, the majority of them are non-viable. Thus the same leucocytic material may be a destructive agent when it stimulates premature subdivision,

<sup>1</sup> *Brit. Med. Journ.* II. p. 296, 1922.

as with bacteria, and a helpful agent when it stimulates the normal growth of animal cells either *in vitro* or *in vivo*.

It must, of course, be remembered that leucocytes are by no means the only cells in the body from which stimuli to growth and to autolytic change can be derived.

*Doubtful enzymes.*

It is always a satisfaction to be able to deal with something which is more or less definitely concrete, something which can be put into a test-tube and bear a name descriptive of its essential property. This has been the case, at least to some considerable extent, with leucocytic enzymes.

Now I come to some more obscure factors about which I desire to raise the question:—May not the true explanation of some doubtful immunological principles be ultimately found in terms of enzymes rather than, as I have suggested above, in terms of cell stimuli?

There is the view that natural immunity against particular bacteria is due to normal secretions of the animal body which act as enzymes destructive to the bacteria, and that the partial resistance of the susceptible animal is due to the same enzymes, which are present, though not in sufficient concentration to be completely effective. Then acquired immunity (active or passive), in the obscure cases where it cannot be explained by antibodies, would be attributed to a stimulus (of bacterial protein or of immune serum) which causes the body to secrete these enzymes in greater quantity.

These ideas would coincide, in the main, with Preisz's way of explaining anthrax immunity. He came to the conclusion, as I have already mentioned (p. 331), that all normal animals, even the most susceptible, possessed in their tissue juices material, probably of leucocytic origin, which was antagonistic to the anthrax bacillus. But this material differed in quantity and concentration in different animal species. If there was enough to kill the bacilli promptly, the animal was immune; if there was less than this amount, some of the bacilli remained alive until they formed capsules; with this protective envelope, they were more resistant against the animal's bactericidal substances and produced a general infection. The essential property of an immune serum, in his opinion, was simply to stimulate in the animal body the production in greater quantity of those bactericidal substances which all normal animals possessed in greater or less degree.

He arrived at these opinions by the method of exclusion, first showing that there were no recognisable antibodies which would explain anthrax immunity. I need not recapitulate here his evidence upon these points, but I think it is interesting to describe his way of investigating the question whether immune serum had the property of increasing the production of bactericidal substances in the animal body.

For this purpose, he inoculated mice subcutaneously on three successive days with 0.5 c.c. of immune serum and, after the last injection, introduced a silk thread under the skin. After the threads had remained there for 24 hours, he soaked them in a broth culture

of anthrax and then inserted them into fresh mice. Control threads were kept for 24 hours in the subcutaneous tissue of normal mice and were then infected with culture and inserted into fresh mice. No difference was found microscopically between the cellular contents of the immune serum threads and the control threads. The controls produced fatal anthrax in the usual way. The mice which had received the threads from the immunised animals either survived completely or lived considerably longer than the controls.

What was the reason? Fresh threads which had been soaked in either immune or normal serum and then dipped into the culture were fully virulent; so he could exclude the possibility that the threads from the immunised mice had absorbed an effective quantity of the immune serum which those animals had received. He also showed that the blood serum of passively immunised mice did not possess bactericidal properties. Anthrax bacilli grew in it freely, and a thread, which had been soaked in it for 24 hours and then infected, was fully virulent.

Preisz completed this series of experiments with some tests *in vitro*. Threads which had been kept in the tissues of passively immunised mice were broken up and incubated in a broth suspension of young anthrax culture. In some of these tests the bacilli were killed, though in others no bactericidal effect was demonstrable. As the contents of the threads (before they were infected) consisted mainly of leucocytes, he thought that their bactericidal action might be explained on the hypothesis that these leucocytes had been stimulated by the immune serum to excrete bactericidal substances.

His general conclusion, as I have already indicated, was that the essential function of anti-anthrax serum in the animal body was to stimulate increased production of normal antibacterial substances.

I am not prepared to disagree with this line of thought. There are plenty of mysterious enzymes in the animal body and many of them may be antibacterial, though it is not necessary to suppose that those which possess this property are all derived from leucocytes. In these doubtful questions it is impossible to assert that a particular influence is or is not due to an enzyme as distinct from what I have called a "stimulus." I think the possibility of the latter kind of influence ought to be borne in mind, but I have no desire to claim for it an exaggerated importance.

If Preisz's experiments with silk threads had been done at the present day, they might perhaps have suggested to him that the process was similar to that described by Bordet in the initiation of a transmissible bacterial autolysis by means of leucocytic exudates. It is tempting to think that the threads taken from the immunised animals contained lytic substances, though it is not clear whether the leucocytes adherent to the threads or some product of interaction between serum and leucocytes provided the condition necessary for stimulating a bacterial autolysis.

Will enzymes explain, or partially explain, another obscure factor? I refer to certain experimental results which Morgenroth and Abraham<sup>1</sup> have attributed to what they call "depression" immunity. Working on antistreptococcal immunity, which they were unable to explain as being entirely due to bacteriotropic action, they made some observations on mice which are suggestive, though one would like to see them corroborated by a larger series of experiments.

The mice were inoculated intraperitoneally with 0.5 c.c. of undiluted antistreptococcal serum, a dose which was found to confer a considerable amount of protection. Three hours

<sup>1</sup> *Zeitschr. f. Hyg. u. Infektionskrankh.*, C., p. 323, 1923.



later, they received an intravenous injection of streptococcus culture in doses which were all rapidly fatal for the controls.

The interesting feature of the experiments was the repeated fluctuations in the bacteriaemic condition. At a period of from 2 to 5 days from the initial bacteriaemia, the cocci would almost, or entirely, disappear from the blood stream; on about the 5th or 6th day, there would again be well marked bacteriaemia, persisting for about a day and then again disappearing; on the 8th day, three of the animals showed a third bacteriaemic phase, which declined on the 9th day; and in one case a fourth bacteriaemic rise was found on the 12th day.

It was also noted that the original haemolytic and highly virulent streptococci became rapidly attenuated and that sometimes there was a reversion to the virulent type in the second bacteriaemic phase.

As the authors point out, direct action of the immune serum on the cocci (after the manner of an ordinary antibody) would not suffice to explain these data. In their view, the function of the serum is to retard the invasive properties of the cocci for a brief period, during which the animal is enabled to bring its own defensive mechanism into play. They regard the first disappearance of the bacteriaemic phase as a manifestation of a special kind of active immunity (*viz.*, "depression" immunity), superimposed upon the influence of the serum. The second bacteriaemic rise coincides with the disappearance of the primary action of the serum and, if no other factors were operative, would speedily be followed by the death of the animal. But what actually happens is a regular and prompt decline in the bacteriaemia, due to a new manifestation of active immunity. This condition is not attributable to a new formation of ordinary antibodies, because, if it were, it would persist. On the contrary, it is replaced on the following day by a renewed bacteriaemia, which may again disappear and afterwards reappear. All these periodic oscillations of resistive capacity they regard as characteristic of "depression" immunity.

Thus, in the authors' opinion, "depression" immunity and attenuation of virulence are factors of essential importance and must supplement the conception that immunity is due to phagocytosis promoted by the action of a bacteriotropic antibody.

I think it may be accepted that the data provided by Morgenroth and Abraham cannot be explained in terms of Ehrlich's antibodies. But the significance, in these experiments, of what they call "depression" immunity is an open question.

I do not think it has been proved that the leucocytes have nothing to do with the observed states of "depression." Even if it be conceded that immunity is not satisfactorily explained by phagocytosis of cocci which have been sensitised by a bacteriotropin, there are still other possible functions of leucocytes to consider.

One cannot afford to ignore the old work on the liberation of antibacterial enzymes by disintegrated leucocytes. Is it unreasonable to postulate that, in streptococcal infections such as these, this liberation may occur in successive waves, with a quiescent interval between each of them? On this view, there would be phagocytosis of sensitised or partially sensitised cocci during the first phase of bacteriaemia and many leucocytes would be killed and disintegrated during this process; the enzymes liberated from them would promptly reduce the bacteriaemia; when these enzymes were exhausted, the cocci would reappear; there would again be phagocytosis followed by release of enzymes from broken up leucocytes; and so the process might continue,

with a reproduction of the fluctuations said to be characteristic of "depression" immunity.

Thus, it seems to me, enzyme action might go a long way towards explaining one of the striking features of these experiments.

The other feature to which Morgenroth and Abraham call special attention—attenuation of the streptococci—I should be inclined to explain as due to a stimulant action. One knows that material from disintegrated leucocytes may initiate a transmissible bacterial autolysis; it may also exert a less profound action, causing the splitting off of variants which are still viable but attenuated. And oscillations in this attenuating influence may perhaps be correlated with fluctuations in the output of the leucocytic material.

But is that all the explanation, or have I been attributing too much to the leucocytes? These are not the only cells in the animal body with which bacteria are concerned; the products and secretions of other cellular constituents may also have an important influence on bacterial growth.

And in other infections where resistance to reinfection is notable, as in syphilis and tuberculosis, it seems quite impossible to attribute the observed phenomena to the activities of leucocytes or any other special type of cell. The same remark probably applies to the grafting of malignant disease, where a similar resistance to reinfection has been observed. The condition, in each case, may be thought to be due to some systemic change affecting the animal organism as a whole.

The operation of some sort of systemic change seems also to be indicated in the earlier work by Morgenroth and his colleagues on "depression" immunity which I have discussed in a previous report<sup>1</sup>.

They observed that mice injected with streptococci of low virulence ("green") were more resistant than normal mice to infection with highly virulent haemolytic streptococci. The results seemed to agree, whether the inoculations were made subcutaneously, intraperitoneally, or intravenously; the prepared mice survived the dose of virulent streptococci (which it was not necessary to inject by the same method as in the preliminary inoculation) longer than the controls which had not received the less virulent organisms. It was noticeable that this "depression" immunity manifested itself very promptly. In some experiments it was definitely evident in 6 hours and was fully developed in 24 hours. The condition was not permanent, apparently owing to a remission in the course of the initial infection. It remained well developed up to the end of the 4th day, but, when the "super-infective" dose was administered on the 5th day, the greater number of the mice succumbed to acute infection, and, when the test was made on the 7th day, almost all the animals died, like the controls, in 24 hours. Cultures from the organs of mice which had exhibited "depression" immunity showed that the growth of the haemolytic streptococci had not been inhibited; it was often found that these organisms were present in very large numbers, greatly in excess of the number in the control mice which had died from acute infection. Another interesting fact was that these haemolytic streptococci recovered from the super-infected mice were fully virulent when tested on normal mice.

For the production of this systemic change with such rapidity, it seems necessary to suppose that the bacterial constituents or products act directly

<sup>1</sup> Ministry of Health. *Reports on Public Health and Medical Subjects*, No. 22, pp. 11–12, 1923.

on the normal elements of the animal's plasma and at once modify their "combining affinities" for the surface of streptococci. A similar explanation has been suggested for the equally prompt and more profound action of anti-anthrax serum. This, it appears, does not combine with the bacilli but acts indirectly, by producing a systemic change; *i.e.* the serum promptly interacts with normal elements in the plasma of the infected animal and causes them to assume "combining affinities" for the surface of anthrax bacilli.

### *Antibodies.*

Certain properties of antibodies bear no resemblance to cell stimuli. The lysins lead to the direct destruction of bacteria by enzyme action; the bacteriotropins or opsonins prepare them for ingestion by the phagocytes; agglutinins may be adsorbed by the bacterial surface without exercising any influence on vital processes of the bacterium. In these instances, the bacterium is not stimulated; it is merely passive.

Here I am concerned with instances where antibodies are also stimulants and are associated with the production of bacterial variants.

I think the most useful example is the production of pneumococcal variants by growth in immune serum, as described by F. Griffith<sup>1</sup>. Here the influence which produces the variant can be identified without hesitation; it is the action of the specific "type" antibodies on the growth of the homologous strain. The variant is viable *in vitro* but not *in vivo*; and its loss of virulence in the animal body is shown to be due to loss of those elements in bacterial structure which are associated with capsule formation and production of "specific soluble substance." Hence the action of the serum in protection tests is readily explained; the serum produces a variant which, owing to these defects, is not viable in the animal body.

A feature of special interest in this work is that the stimulant action is found to be strictly confined to "type." Types I, II and III, being all pneumococci, must have many properties in common; but Type I serum will only act as a stimulant with Type I strains, and similarly with the sera and strains of Types II and III.

I called attention to what I regard as similar phenomena in a former report<sup>2</sup> in which I discussed immunological reactions with antigens artificially prepared by the introduction of a known chemical component. In some cases the new component has the effect of narrowing down the range of the antigen-antibody reactions. I quoted an example given by Pick.

It was found that an antiserum prepared by immunising with diazobenzol-oxprotein would precipitate only diazobenzol-oxprotein, not normal oxprotein nor the protein of man, horse, or dog, which had been turned into the diazobenzol compound.

In general terms, when an antigen A (here the serum of a particular species of animal) is linked to a new antigenic character *b* (here a known

<sup>1</sup> Ministry of Health. *Reports on Public Health and Medical Subjects*, No. 18, 1923.

<sup>2</sup> *Ibid.* No. 13, p. 49, 1922.

chemical compound), the combination  $Ab$  may retain its original specificity (due to  $A$ ) whilst acquiring a new "constitutive" specificity (due to  $b$ ); thus the antiserum produced by immunising with  $Ab$  may react only to  $Ab$  antigen, not to  $b$  coupled with an antigen other than  $A$ , nor to the normal  $A$  antigen, nor to  $A$  coupled with  $c$  or  $d$  or  $e$ , etc.

Similarly with pneumococci,  $A$  being their common species characteristics, and  $b$ ,  $c$ ,  $d$  etc. the characteristics peculiar to each type. The immune sera stimulate the production of variants in a highly specific and selective manner which is determined by "type" characteristics.

With other bacterial species quite different results might be found. The immune serum obtained by treatment with a single strain might act on all or several different "types" of the species and might also have some action on other species. For the existence of such differences there is no satisfactory explanation; they have to be accepted as facts, which, in connection with the present subject, coincide with the haphazard characteristics of cellular stimulants.

I now come to another curious point in relation to the specificity of stimuli (as demonstrated by antibody reactions) which it is equally difficult to explain.

Such stimuli may initiate transmissible bacterial autolysis. For example, a stimulus  $a$  (derived from bacillus  $A$ ) may cause a normal culture of bacillus  $C$  (an organism different from  $A$ ) to produce lytic substance  $c^1$ , which may be transmitted to a fresh normal culture of  $C$ , and so on, indefinitely. Another stimulus  $b$  (derived from  $B$ , an organism different from both  $C$  and  $A$ ) may produce the same effect on  $C$ , yielding a supply of lytic substance  $c^2$ , which may be propagated *ad infinitum*. One might suppose that, after several transmissions through identical cultures of normal bacillus  $C$ ,  $c^1$  and  $c^2$  would be completely identical with each other. But this may not be the case. If the lytic properties of the propagated  $c^1$ , and  $c^2$  be tested on a culture of  $A$ , it may be found that  $c^1$  produces lysis whilst  $c^2$  fails to do so.

How does  $c^1$  "remember" its affinity for  $A$ ? Did  $a$  cause the protoplasm of  $C$  to break up in a special way, *viz.*, into particles containing affinities for both  $C$  and  $A$ ? And was this special mechanism of autolysis perpetuated in the transmission of  $c^1$  from culture to culture of  $C$ ?

In a previous report<sup>1</sup> I quoted two examples in which this peculiar "remembrance" of a former state was demonstrated by immunising animals with a lytic substance and obtaining in their serum the corresponding specific antibody.

(1) Bruynoghe and Appelmans<sup>2</sup> prepared antisera with two typhoid "bacteriophages," the one obtained from Strasbourg and the other from Louvain. The latter was originally a coli "bacteriophage" but had been adapted to the typhoid bacillus and had lost all action on coli. It was found that its antiserum neutralised not only the Louvain "bacteriophage"; but also its predecessor, the coli "bacteriophage"; it had no action on the Strasbourg "bacteriophage." The Strasbourg antiserum neutralised only the Strasbourg "bacteriophage."

<sup>1</sup> Ministry of Health. *Reports on Public Health and Medical Subjects*, No. 18, pp. 31-2, 1923.

<sup>2</sup> *C.R. Soc. Biol.* LXXXVII, p. 96, 1922.

(2) Gratia and Namur<sup>1</sup> obtained somewhat similar results with antisera for substances which were lytic for staphylococci. The one substance (BH) was lytic primarily for *Staphylococcus aureus* (H) and the other (BV) for *Staphylococcus albus* (V); but, whereas BH acted on many strains of these organisms, including V, BV was strictly specific for V. The antiserum prepared with BH was specific for BH alone and the antiserum for BV was equally selective for BV. After the lytic principle BH had been allowed to act on V and had been transmitted ten times through V, the action of the two antisera was tested on the lytic substance finally obtained. It was found that no change had taken place; specific neutralisation was still obtained by the BH antiserum and not by the BV antiserum.

Is there any use in attempting to explain these apparent instances of a peculiar "remembrance" or "inheritance" of a particular affinity? In natural immunity it has to be admitted that it is useless to attempt impossibilities; the constituents of the plasma are too complex for analysis and one cannot single out any particular element, present in the naturally immune and absent in the naturally susceptible animal, which has a combining affinity for the bacterium in question; one can only assume that such differences exist.

But in the instances referred to above it may seem possible to go a little way towards the analysis of the experimental data, because it would appear to be a relatively simple question of the particular combining affinities possessed by certain lytic substances and their antisera. One method would be to postulate the presence of two constituents in the lytic substance, one (A) of which is "dominant" (*i.e.*, free to participate in chemical reactions), whilst the other (B) is "masked" (*i.e.*, is linked up with other chemical groups in such a way that it cannot directly participate in fresh chemical reactions). Thus a lytic principle, though containing both A and B, may be neutralised by an anti-A serum but not by an anti-B serum.

I am not prepared to agree that this is the right explanation. My main reason for mentioning it is to call attention to the dangers of adopting this method of explanation as a general principle. If one starts applying it first to one particular instance and then to others, one soon becomes involved in the general postulate of a multiplicity of antibodies, each selective for a corresponding substance in a multiplicity of antigenic constituents. Even as regards true antigen-antibody reactions, there are reasons, which I discussed in a previous article<sup>2</sup>, for thinking that this postulate is untenable. And, in so far as the substances which act as antibodies also act as stimulants, it is a postulate which cannot possibly be maintained, since it would be absurd to assume that for each special kind of stimulative effect there is a special and chemically distinctive kind of stimulus. The examples from the idiosyncrasies of pneumococcal types are the exception, not the rule.

The above considerations make one pause before attempting to form some sort of simple mental picture which would help to explain why an antibody, *i.e.*, (1) a participant in an antigen-antibody reaction, can also act as (2) a stimulus.

The easiest conception would be that the same combining group in the

<sup>1</sup> *C.R. Soc. Biol.* LXXXVII. p. 364, 1922.

<sup>2</sup> *Journ. Hyg.* XXII. p. 355, 1924.

antibody is operative in each case and combines with the same specific material, the differences being due to the after-effects of the combination. In (1) it unites with some chemical group attached to the bacterial protein and either it remains united or, if union is followed by dissociation, this release leaves an intact bacterium and an intact antibody. In (2) there is union, as before, with a specific "building-stone" at the surface of the bacterium, but this union with the bacterium is promptly followed by dissociation; the "building-stone," however, being more firmly attached to the antibody than to the bacterium, is carried away from the latter; this interference with the normal process of bacterial metabolism leads to the production of a variant.

This kind of explanation might pass muster for variants amongst pneumococcal types, but I am afraid that, in reality, the action of immune bodies is often much more complicated, because each of them usually possesses a wide range of different combining affinities and these cannot be explained on the "mosaic pattern" postulate that each immune body is really a "mosaic" of different antibodies. I may quote in illustration some recent observations of Landsteiner and van der Scheer on precipitins<sup>1</sup>:—

"The results of precipitin reactions with antigen containing binding groups of known chemical constitution leave no doubt as to the fact that a single precipitin will regularly react with other substances if their chemical structure is sufficiently near to that of the homologous antigen. The alternative idea, so widely accepted,—that a given immune serum which reacts on the homologous antigen A and also on antigen B derived from another species consists of antibodies specific for a common group of A and B and contains other antibodies specific for a group peculiar to A,—is not tenable in this case."

### *Nutrition.*

It is easy and frequently useful to draw a distinction between a stimulus and a food. The former causes the cell to function in a particular way but is not incorporated as part of the structure of the cell; the latter is utilised by the cell as a "building-stone."

At the same time it must be remembered that it is often impossible to draw a hard and fast line of demarcation between the two. Some chemical or physical agency may alter the characters of the available food supply, making the cell's task of assimilation either easier or more difficult; such an agency cannot be classed either as a stimulant or as a food, though it indirectly serves both purposes. In the symbiosis of bacteria, the products of bacterial action which help other bacteria to grow may be more than merely stimulative; some of them may become synthesised in bacterial cells. A bacterium may, to begin with, be unable to break up and utilise a particular substance, *e.g.*, lactose; but it may, by repeated subculture in a medium containing lactose, be "trained" to ferment this substance; the lactose, then, has acted first as a stimulant and afterwards as nutrient material.

I need not multiply instances. No confusion need arise if one remembers

<sup>1</sup> *Journ. Exp. Med.* XL. p. 91, 1924.

that stimulants, enzymes, antibodies, and food material are not necessarily separate entities, and that one substance may exercise more than one function.

#### SUMMARY.

1. Immunity cannot be completely explained by antigen-antibody reactions, even if the term "antibodies" be made sufficiently elastic to include various obscure properties which are exhibited, *in vivo*, in the actively immune animal. Various other factors have to be considered. One of these is the influence of stimuli upon the vital capacities of bacteria.

2. Transmissible bacterial autolysis appears to be due to a stimulus acting upon the growing bacterial cell and leading to the splitting off of a certain number of daughter-cells which are non-viable, and consequently undergo autolysis.

3. Transmissible autolysis is not due to a stimulus *sui generis* but is no more than a particular incident in the general phenomena of bacterial variation.

4. The secretions of bacteria in pure culture may stimulate, control, or retard their growth and may lead to the production of variants.

5. When introduced into the animal body, bacteria encounter stimulants of animal origin which may be either favourable or unfavourable to their growth and are to be distinguished from the stimulants attributable to the bacteria themselves.

6. One aspect of the differences between natural immunity and natural susceptibility may be interpreted as due to differences in the stimuli inherent in the particular animal species and to consequent differences in their effects upon the particular bacterial species.

7. Similarly, when no better explanation is available, the acquired immunity (active or passive) of a susceptible animal may be interpreted as a change of the animal's stimulant action from one which was favourable (or indifferent) to the growth of a bacterium to one which is adverse, *i.e.*, a stimulant to the reproduction of daughter-cells which are non-viable in the animal body.

8. Leucocytes are one of the sources of material possessing two kinds of properties, *viz.*, (*a*) stimulant action on the growth capacities of cells (both bacterial and animal) and (*b*) enzyme action on the constituents which living and dead cells possess in common. The older researches on the characters of leucocytic extracts were occupied with (*b*), though they may occasionally be linked up with (*a*), since there are some indications that their leucocytic material was also acting as "lytic substance."

9. In some cases it must remain, for the present, an open question whether demonstrable antibacterial action is attributable to some more or less obscure enzymes or to what I have termed "stimuli."

10. A stimulus may be a substance which is also an antibody, and its stimulative properties may be highly specific. But it would be absurd to assume that for each special kind of stimulative effect there is a special and

chemically distinctive kind of stimulus. Both stimuli and antibodies usually possess a wide range of different combining affinities which cannot be explained on the "mosaic" theory that each different combination is due to the presence (in the stimulus or antibody) of a different chemical group.

11. A stimulus, as distinct from a food, causes the bacterial cell to function in a particular way but is not incorporated as part of the structure of the cell. This convenient distinction, however, does not imply that there is necessarily a sharp line of demarcation between a stimulus and a food.

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## MELIOIDOSIS AND ITS RELATION TO GLANDERS.

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(With Plate X.)

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## 1. INTRODUCTION.

MELIOIDOSIS bears such a striking resemblance to glanders, that Colonel Whitmore, who was the first to recognise this disease, described it under the title of "A glanders-like disease occurring in Rangoon" (Whitmore, 1913). When he first met with the disease in the post-mortem room, he thought that he was dealing with glanders. It was not until he discovered that the lesions were associated with the presence of a strange organism that he concluded that he had encountered a new disease.

The pathological features of melioidosis and glanders are almost, if not quite, identical. In both diseases there are pyaemic nodules which have a tendency to caseate in some organs and to suppurate in others. In both diseases the minute structure of the nodules is the same. McFadyean states that the two distinguishing characteristics of the glanders nodule are, first, the peculiar degeneration, or chromatotaxis, which occurs in the centre of it, and secondly, its slight tendency to peripheral extension. These features are equally characteristic of the melioidosis nodule, and it is difficult if not impossible to distinguish one disease from the other by means of inspection with the naked eye or by the examination of sections under a microscope.

The two diseases resemble each other clinically almost as much as they do in their morbid anatomy. The first case of melioidosis to be observed throughout its course was under the care of Major Knapp in the gaol at

Rangoon. The symptoms were like those of glanders, and it was only when a bacteriological investigation was made, after death, that the true nature of the malady was disclosed (Whitmore, 1913).

Knapp (1915) suggested that the new disease might be distinguished from glanders by the absence of skin lesions, but these occur in both diseases; bullae and pustules have been present in several of the cases which we have seen in Kuala Lumpur. Pustular rashes, abscesses in the subcutaneous tissue, in the muscles and in the bones are features of the later stages of melioidosis as well as of glanders. Both diseases may resemble malaria, acute rheumatism, enteric fever or tuberculosis and, in the early stages, diagnosis may be impossible.

On comparing our experience of melioidosis with the text-book descriptions of human glanders, we find that there is a difference in the usual course of the two diseases. Melioidosis is more often acutely fatal. The majority of our patients died within a period of ten days after being attacked, and nearly all Whitmore's cases must have died very shortly after the onset of the disease, because they were either dying or already dead when they were brought to the hospital.

When animals become infected, nasal discharge is a common symptom in both melioidosis and glanders; nodules are often present in the nasal septum, and the lungs are almost invariably diseased.

Chronic melioidosis is rare in man and it has not been possible to apply the mallein test in more than one case. In this instance it produced a positive reaction. We obtained a supply of mallein through the kindness of the Director of the Geneeskundig-Laboratorium at Batavia. This was diluted with nine parts of salt-solution and 0.2 c.c. were injected into the skin of the forearm of a patient who had been suffering from melioidosis for two years. In less than two hours his temperature had risen to 100.8° F. On the following day, eighteen hours after the inoculation, the temperature was 99° F. and there was a large, red, diffuse, tender swelling, at the site of inoculation, which measured 10 by 6 cm. On the third day, forty-two hours after inoculation, the temperature was 99.6° F.; the swelling was larger and the patient complained that pain had kept him awake during the previous night. The fever and swelling then subsided and, within ninety-six hours of the time at which the injection was given, all signs had disappeared. Four controls were inoculated with entirely negative results.

Mallein tests in laboratory animals did not prove satisfactory. Two rabbits, which had been immunised with killed cultures of a strain of *B. mallei* obtained from Java, were inoculated with 0.02 c.c. of mallein, and in each of them there was a slight reaction in the form of a small, red papule which lasted for about three days. Another rabbit which had been immunised with a culture of *B. whitmorei*, the causative organism of melioidosis, gave a similar result. None of these reactions was nearly so definite and pronounced as in the human case.

A guinea-pig immunised with killed cultures of *B. mallei*, Java, did not react to mallein. Two guinea-pigs with abscesses resulting from injections of living *B. mallei* gave no reaction. Three guinea-pigs with large abscesses due to injections of *B. whitmori* failed to react with mallein.

The intimate relationship of melioidosis and glanders is clearly demonstrated by the presence of agglutinins for *B. mallei* in the blood of persons suffering from chronic melioidosis. The blood of the man, Ragaviah, who gave the positive mallein reaction, agglutinated *B. whitmori* (cultivated from his own lesions) in a dilution of 1 in 4000. Tested with three strains of *B. mallei*, it agglutinated two of them (strains "Muktesar" and "Java") up to 100 per cent. and the other strain "Minett" up to 10 per cent. of the full titre. In addition, one of these strains, *B. mallei* "Muktesar," absorbed the agglutinins for the homologous *B. whitmori*.

As we have seen, there is little or no difference in the morbid anatomy of glanders and melioidosis, and the difference in their clinical course lies more in the greater pathogenicity of the latter infection than in its fundamental characters. Their epizootology, however, appears to be quite distinct; both are primarily diseases of animals and rarely affect man; but while glanders is an equine disease, melioidosis is a disease of rodents (Stanton and Fletcher, 1921). There are very few horses in the Federated Malay States and, in those cases of melioidosis where it has been possible to investigate this point, there has been no opportunity of infection from these animals.

Apart from other differences in the two diseases, it was because the organisms which caused them appeared so totally unlike one another on cultivation outside the body that Whitmore decided that his cases were examples of a new disease caused by a new bacillus. He (Whitmore, 1913) stated that the cultural and other characteristics, upon which he relied for distinguishing this bacillus from other pathogenic bacteria, were as follows:

1. Rapid and luxuriant growth upon ordinary peptone agar.
2. The wrinkling which occurs so early in the growth upon glycerine agar.
3. The pellicle formation at the surface of broth cultures.
4. The appearance of gelatine stab cultures at the end of the third day, and after one week's growth.
5. The curious, tangled masses of long filamentous bacilli found in cultures upon salt agar.
6. The active serpentine motility of the bacilli in young cultures and its disappearance as the cultures age.

In order to determine if these criteria are fulfilled by the bacillus of glanders as well as by Whitmore's organism, we obtained five strains of *B. mallei* which we compared by means of cultural and serological tests with fourteen strains of *B. whitmori*.

The sources of the five cultures of the glanders bacillus were as follows:

- (1) *B. mallei*, Java. This strain was kindly sent to us by the Director of the Geneeskundig-Laboratorium at Batavia, in Java, on August 8, 1922.

It had been isolated from the lungs of a country-bred pony, more than a year previously, and had been passed through a number of guinea-pigs.

(2) *B. mallei*, Muktesar. A culture of this organism, from the Imperial Bacteriological Laboratory at Muktesar, in India, was received in September, 1922. Dr J. V. Edwards, the bacteriologist, kindly informed us that this strain was isolated from a country-bred pony and had been maintained for a long time in culture and by passage through guinea-pigs.

(3) *B. mallei*, Minett. This strain, and the two which follow, are from the National Collection of type cultures, and were supplied through the kindness of the Director of the Lister Institute. The Minett strain was isolated from the lung of a horse at the Royal Veterinary College in 1918.

(4) *B. mallei*, Egypt, was isolated in November, 1922, by Captain F. C. Minett, in Egypt, from a case of cutaneous glanders in a horse.

(5) *B. mallei* A. is the stock strain of the glanders bacillus at the Lister Institute.

The origin of the fourteen strains of *B. whitmori* was as follows: They comprised ten which had been isolated from human cases and four cultivated from animals naturally infected with melioidosis. One of the human strains, *Finlayson*, was isolated from the lung of a patient in Singapore. The other nine were obtained from cases in Kuala Lumpur. Four of these nine cultures, namely *Govindasamy*, *Pavaday*, *Sellen* and *Vengkatten*, were grown from spleens; two, *Mananay* and *Warton*, were from livers; *Velu* from a lung; the two cultures *Ragaviah* (corrugated) and *Ragaviah* (mucoid) were both isolated from a loopful of pus taken from an abscess in the leg of a patient suffering from chronic melioidosis.

The origin of the four animal strains was as follows: The strain known as *Cat* was cultivated from the spleen of a domestic cat which was brought to the laboratory with melioidosis contracted under normal conditions; probably from eating an infected rat. The strain *Jacques's rat* was isolated from the caseous lungs of a rat which was found dead from melioidosis in the house of a European officer, five miles distant from this laboratory. *Rabbit 45* (corrugated) was obtained from the spleen of a rabbit which had become infected in the animal-house attached to this Institute. *Rabbit 45* (mucoid) was cultivated from the caseating tunica vaginalis of the same animal.

We have adopted the two strains *Ragaviah* (corrugated) and *Ragaviah* (mucoid) as our standard cultures of *B. whitmori*. As mentioned already, both of them were isolated from an abscess in the leg. We have related elsewhere (Stanton and Fletcher, 1921) that *B. whitmori* occurs in two forms; first the more common variety which grows as an opaque culture with a corrugated surface on glycerine agar and, secondly, a mucoid variety which forms a smooth, translucent film on the same medium (see Pl. X). The mucoid variety of *B. whitmori* bears the same relationship to the ordinary corrugated form as the mucoid variety of *B. paratyphosus* bears to the

ordinary form of that organism (Fletcher, 1920). Corrugated colonies can be grown from a mucoid culture, and we have proved the identity of the two types by agglutination and absorption tests. If peptone water is inoculated with the mucoid culture of the "Ragaviah" strain of *B. whitmori* employed in these experiments, and after ten days' incubation at 37° C. is plated out on agar, then about 10 per cent. of the colonies on the plate are of the corrugated type; the corrugated colonies are "thrown off" by the mucoid form and are derived from it. The fourteen strains which were used in the following experiments were all of the common corrugated form except *Ragaviah* (mucoid) and *Rabbit 45* (mucoid).

## 2. THE CULTIVATION OF *B. WHITMORI* AND *B. MALLEI*.

In peptone water the mucoid strains of *B. whitmori* are indistinguishable from glanders cultures, except that growth is more vigorous in the former; but the appearance of the corrugated form of *B. whitmori* is quite different from its own mucoid form and from *B. mallei*. The corrugated strain forms a film on the surface of the medium which becomes a thick, wrinkled pellicle in the course of two or three days. The strains of *B. mallei* which we examined did not possess this property. The culture from Muktesar grows as a film on the surface of peptone water, but this film does not become thick or wrinkled. The other strains of the glanders bacillus produce ropy deposits in the depth of the medium, and the upper part becomes clear. There are no obvious differences in the morphology of the organisms as seen under the microscope in a hanging drop; but the cultures of *B. whitmori* are actively motile and thus they can be distinguished at once from the strains of *B. mallei*, none of which are motile even after daily subculture in liquid media for a period of three weeks. Indol is not formed in peptone water, either by *B. whitmori* or *B. mallei*.

The appearance of cultures of the corrugated form of *B. whitmori* on solid laboratory media is entirely different from that of *B. mallei*. On ordinary agar, *B. whitmori* is white and opaque instead of being slimy and semi-transparent. On glycerine agar it is wrinkled and corrugated, like an old culture of tubercle bacilli, whereas *B. mallei* is smooth and shining (see Pl. X). Cultures of the mucoid form of *B. whitmori* on solid media are not unlike *B. mallei*, but the growth of the former is more vigorous and the diagnosis can be speedily settled by inoculating a tube of peptone water and examining the culture for motility eighteen hours later.

The growth of the corrugated form of *B. whitmori* on potato is quite different from all the other cultures investigated. It is a dull, dirty white, not shining and yellow like *B. mallei*. The mucoid forms of *B. whitmori* on potato are indistinguishable from *B. mallei*.

The difference between *B. whitmori* and *B. mallei* in their action on carbohydrates is one of degree. *B. whitmori* ferments glucose; the surface of glucose peptone-water becomes pink, in about thirty-six hours, when Andrade's indicator is employed. The colour soon spreads through the medium, reaching

the Durham's gas-tube last of all. Bleaching of the indicator commences on the fourth or fifth day and proceeds in the same order, being complete by about the tenth day. Mannite and dulcite show a pink tinge at the surface in four or five days, but the process goes no further. Lactose and saccharose are not fermented by strains which have been subcultured repeatedly in the laboratory. No gas is produced in any medium.

The fermentation reactions of the bacillus are more vigorous when it is first isolated from the body. The first subculture of the strain *Warton*, which was isolated from a human liver in December, 1923, fermented glucose and saccharose in twenty-four hours and, in seventy-two hours, it fermented lactose, mannite and dulcite as well. Two months later, in February, 1924, it still fermented glucose, mannite and dulcite but not lactose or saccharose. In March, it fermented glucose as before, but its action on mannite and dulcite had become exceedingly feeble.

All the strains of *B. mallei*—Muktesar, Java, Minett, Mallei A. and Egypt—ferment glucose in seven to thirty days. The medium remains red; the indicator is not bleached, as in the case of *B. whitmori*. Lactose, saccharose, mannite and dulcite are not fermented by *B. mallei*.

*B. whitmori* has a more destructive action on milk than *B. mallei*, but here again the difference is only one of degree. Litmus milk inoculated with *B. whitmori* becomes acid in three days. In five days it separates into a fatty curd on the surface, an intermediate layer of dirty, brown whey, and a granular, buff-coloured deposit at the bottom of the tube.

Milk inoculated with *B. mallei* becomes acid in about the same length of time, but it does not clot until the tenth day. The clot is bright pink above, and white below. On the surface of the medium there is a bright, pink, creamy layer. The clot is not digested, but it contracts and exudes a colourless whey.

Gelatine is liquefied very rapidly by *B. whitmori* and very slowly by *B. mallei*. All the strains of *B. whitmori* which we examined commenced to liquefy gelatine on the fourth or fifth day, at a temperature of 20° C.; but none of the *B. mallei* cultures grew well at this temperature and none liquefied gelatine. At 37° C. gelatine is completely liquefied by *B. whitmori* in four days. That is to say, it is digested and does not become solid when placed in the refrigerator. Liquefaction, at this temperature, occurred on the thirty-sixth day with *B. mallei*, Minett; on the fortieth day with *B. mallei*, Muktesar, and on the twenty-sixth with *B. mallei*, Java.

It is impossible to distinguish *B. whitmori* from *B. mallei*, with certainty, by the examination of stained films. Formerly we considered that bipolar staining with Leishman's stain was a distinguishing feature of *B. whitmori*, but, in preparations made from peptone-water cultures, we found bipolar organisms in all three cultures of *B. mallei* as well as in those of *B. whitmori*.

In films prepared from cultures on ordinary agar, stained with Leishman's stain, many strains of the corrugated form of *B. whitmori* consist almost

entirely of deeply staining, bipolar organisms. There are also some bipolar bacilli in the cultures of *B. mallei* from Muktesar and Java, but they are not so numerous, nor do they stain so deeply. The Muktesar strain contains long hyphae, some as long as 70 micromillimetres, which break up into bipolar-staining bacilli. These hyphae are particularly remarkable in the ropy deposit which is found at the bottom of glucose-broth tubes, inoculated with *B. mallei*. In the case of the Java bacillus some of these long forms are beaded and branched like moulds.

Whitmore lays stress upon the long strings and chains formed by the bacillus on 2 per cent. salt-agar. This phenomenon is more common with the corrugated than with the mucoid form, but it cannot be relied upon, because it is not a constant feature of *B. whitmori*, and also because *B. mallei* sometimes grows in a similar fashion.

### 3. MELIOIDOSIS IN ANIMALS.

Melioidosis has been present among our stock of laboratory animals, first as an epidemic and later in sporadic form, for nearly eleven years. An outbreak which attacked the rabbits and guinea-pigs occurred at the end of 1913. A large number died and, in addition, a couple of rats which had been caged for experimental purposes became infected and succumbed to the disease. The earliest symptom in rabbits and guinea-pigs was a white milky discharge from the eyes and nose; within a few days the breathing became difficult and the animal died. Every case was fatal. The appearance found post-mortem depended very largely upon the duration of the illness.

In animals which had died very quickly from acute septicaemia, the only visible signs of disease, apart from the milky discharge about the eyes and nose, were congestion of the nasal mucous membrane, trachea and lungs, and a few, yellow, miliary nodules on the nasal septum. As a rule the bladder was noticeably distended with urine; sometimes this distension was so great that the top of the bladder reached the costal margin, and in one rabbit, of small size, the contents measured more than half a pint.

When the course of the disease was slower the lungs contained minute, caseous nodules, like miliary tubercles which consisted of a bronchiole and its surrounding alveoli filled with nuclear debris. In animals which lived still longer the lungs were consolidated, the swollen and detached epithelial lining blocked the bronchi, in places all alveolar structure was lost, while here and there small haemorrhages were seen. In the two rats, where the course of the disease was far less acute than in rabbits and guinea-pigs, the miliary nodules had spread and coalesced until the thorax was filled with a caseous mass firmly adherent to the thoracic walls. Very little lung tissue remained which was not caseous; in the caseous mass the heart was embedded, and the oesophagus behind it was studded with minute caseating nodules.

The characteristic lesion of the disease is a small, yellowish, caseous nodule. These nodules were found in almost every part or organ of the body and they

sometimes coalesced to form large caseous masses. *B. whitmori* could be cultivated from them in whatever situation they occurred and it was usually found in films made from the smaller nodules though not often present in very large numbers.

Histologically, as in several other respects, melioidosis resembles glanders. Both diseases are characterised by the formation of nodules which commence as small collections of polynuclear cells; these subsequently necrose, but their chromatin persists and retains its affinity for nuclear stains. This persistence of chromatin fragments is so striking a feature of the glanders nodule that Unna coined a special name for it, "chromatotaxis."

The spleens of our naturally infected laboratory animals were always enlarged. Except in very acute septicaemic cases they were speckled, like the spleens of plague rats, with small nodules of focal necrosis composed of broken down nuclei embedded in necrotic caseous material. Melioidosis usually runs a more chronic course in native rats (*M. griseiventer*) than in guinea-pigs and rabbits. In rats which have died from melioidosis the spleen sometimes contains large caseous nodules very like those found in the spleen of a rat with resolving plague.

The hearts of two of the rabbits contained specific lesions in the form of caseous nodules, the size of split-peas, adherent to the endocardium in the left auricle. These nodules consisted of fibrin and nuclear debris invaded by small cells and blood vessels. Fluid was sometimes found in the pericardium and, in a few guinea-pigs, the sac was full of caseous material.

In some cases the peritoneum was covered with small tubercles, like the tubercles of miliary tuberculosis, and the great omentum was rolled up and studded with yellow caseous nodules. The liver was affected in about one-eighth of the animals. In one of the rabbits there were nodules in the kidney. The disease frequently attacked the testis and epididymis. The testis was fixed in the scrotum by adhesions, while the epididymis and the tunica vaginalis were represented by a mass of caseous nodules with the usual histological characters. The lymphatic glands in the regions of the jaw, neck and axillae were sometimes enlarged and haemorrhagic, with caseous nodules in their substance.

#### 4. THE INOCULATION OF ANIMALS WITH *B. WHITMORI*.

We attempted to reproduce the disease by inoculating laboratory animals with the organisms isolated from rodents which had become infected naturally, and numbers of rabbits, guinea-pigs and rats were inoculated with these animal strains and also with those derived from human cases of melioidosis.

Large doses of virulent cultures caused death from septicaemia in less than twenty-four hours. The subcutaneous inoculation of smaller amounts was always followed by a local reaction; a hard swelling formed which consisted of coagulated lymph and oedematous necrotic tissue. About the third day a small slough appeared in the middle of the swelling at the site of the



needle puncture; this gradually spread until it formed a crater-like ulcer with ragged, undermined edges and a yellowish slough in the centre. When only a very small quantity of an old laboratory culture was injected, the local lesions healed and the animal lived for two or three weeks. When it died, the lymphatics leading from the point of inoculation were sometimes enlarged and caseous, like farcy pipes, while the scapular and inguinal glands were swollen and filled with thick yellow pus from which *B. whitmori* was easily cultivated. In those animals which lived long enough the organisms were carried by the blood stream to the lungs, spleen and other viscera, where they produced the typical nodules of melioidosis.

Melioidosis, like plague, can be inoculated successfully by scarification. Merely scratching the skin with a knife, contaminated with infective material, is usually sufficient to convey the disease. The skin of the abdomen of a couple of rabbits was lightly pricked with a needle which had been dipped into a culture of *B. whitmori*. One of the animals died on the eleventh day and the other on the thirteenth. Nodules were found at the site of inoculation and in the spleen; but there was no nasal discharge in either of the cases.

All the inoculated animals died, but the respiratory system was not affected to the same degree as it was in the cases which occurred naturally, and in no instance was there any milky discharge from the eyes and nose. It was clear to us that the counterpart of the disease, as we had seen it occur spontaneously in rabbits and guinea-pigs, was not produced by inoculation of the virus into the skin; consequently we turned to investigate the possibility of the infection being conveyed to animals in their food. Fresh vegetable leaves were sprinkled with a culture of *B. whitmori* and fed to rabbits and guinea-pigs. Within two or three days the nose and eyes became moist, soon there was a milky discharge, the breathing became obstructed and the animals died in a week or ten days with all the signs and symptoms of the disease, as we had seen it occur naturally. A plug of cotton-wool moistened with an emulsion of the organism and rubbed on the nose or tongue is sufficient to cause infection.

Strauss's reaction, which is looked upon as a cardinal test for the glanders bacillus, is produced in the same manner by the bacillus of melioidosis. If a small quantity of an emulsion of either organism be inoculated into the peritoneal cavity of a male guinea-pig, within three days the testes become swollen and inflamed; subsequently they are converted with a caseous mass to which the skin is adherent. Owing to the great pathogenicity of *B. whitmori*, a smaller quantity must be injected than when *B. mallei* is employed, otherwise the animal may die in a couple of days from septicæmia.

Rats, cats, monkeys and goats are susceptible to melioidosis. We have seen several rats and one cat which died from the disease contracted under natural conditions; we have infected goats by inoculation and a monkey by mixing a culture of the organism with its food. Rats are more resistant to the disease than rabbits and guinea-pigs. Several rats (*Mus griseiventer*) were

inoculated subcutaneously, one lived for thirty-one days and another for as long as fifty days, after inoculation, before they died with caseous masses in their lungs and other viscera. There was no nasal discharge in the inoculated rats, nor was there any in the rats which became infected naturally.

In a comparative study of *B. whitmori* and *B. mallei* it is obviously of paramount importance to investigate the pathogenicity of *B. whitmori* for horses, and we are indebted to Major S. L. Symonds, B.V.Sc., for procuring animals and inoculating them for us. Five Java ponies and two Australian horses, none of which reacted to mallein, were inoculated with four strains of *B. whitmori* from human cases of melioidosis. Three of these strains had been isolated recently and were very virulent for rodents. In one instance, a thousand million organisms from a glycerine agar slope, inoculated direct with pus from an abscess in the parotid gland of a man suffering from melioidosis, were injected subcutaneously into the neck of a Java pony. Five of the animals were inoculated with saline emulsions of agar cultures and two with broth cultures. One was inoculated with the virus intravenously, one intra-nasally and the rest subcutaneously. The number of organisms injected in each case varied from about one thousand million to ten thousand million. The results were almost entirely negative; none of the horses developed a general infection. In three of them there was a moderate degree of fever which lasted for four or five days. The subcutaneous inoculations produced a localised swelling which suppurated and after discharging some thick pus left an ulcer some 5 centimetres in diameter. This lesion healed in a few weeks and the animal was none the worse.

Three of the horses were killed six months after inoculation and three died from kumrie and old age within eight weeks. No signs of melioidosis were seen in any of them, nor could *B. whitmori* be cultivated from the tissues or viscera. One of the animals remains in good health. We must therefore conclude that *B. whitmori* is not pathogenic for horses, even when it has been newly isolated from a case of melioidosis and is highly virulent for rodents.

#### 5. THE INOCULATION OF ANIMALS WITH *B. MALLEI*.

The strains of the glanders bacillus which were in our possession had been grown for long periods on artificial media and had lost almost all their pathogenicity for animals. Two rats which we inoculated with *B. mallei*, Java, remained in good health; rats of the same species (*Mus griseiventer*) inoculated with similar amounts of laboratory cultures of *B. whitmori* died with chronic melioidosis in two or three months. The latter organism is much more virulent when it is newly isolated; two rats which were inoculated with the first subculture of the "Warton" strain of *B. whitmori*, isolated from a human liver, died from septicaemia in less than twenty-four hours.

Three guinea-pigs were inoculated subcutaneously, one with about four thousand million, and two with two thousand million, *B. mallei*, Java. Each one developed an abscess at the site of inoculation, but they all recovered

and, when they were killed a month later, no signs of disease could be found. A fourth guinea-pig inoculated subcutaneously with two hundred million organisms of the same culture showed neither local nor general signs of disease. Inoculation of a similar quantity of *B. whitmori* invariably causes death.

A rabbit which was inoculated by introducing a loopful of *B. mallei*, Java, into the anterior nares died seventy-two days later from scabies. There were no indications of glanders nor was the bacillus recovered from the organs.

A second rabbit, inoculated with two hundred million organisms of the same culture, developed a small abscess at the site of injection. It died forty-eight days later from intestinal obstruction, but there were no other lesions except the partly healed abscess, and *B. mallei* was not cultivated from the body. A guinea-pig inoculated with a culture of mixed organisms from the abscess remained healthy.

The *B. mallei* strain "Muktesar" had still less effect on the animals inoculated, for it did not produce even a localised abscess. Pledgets of cotton wool moistened with a culture were inserted into the nostrils of a guinea-pig and of a rabbit, but the animals remained in good health. Guinea-pigs and rabbits inoculated in the same way, with cultures of *B. whitmori*, develop melioidosis and die.

A rabbit and a guinea-pig were inoculated subcutaneously, the one with five hundred million organisms and the other with two hundred million, but they both remained healthy. The Muktesar strain of *B. mallei* had obviously become avirulent. A horse inoculated subcutaneously with 1.0 c.c. of a culture in blood-broth, forty-eight hours old, manifested no sign of glanders. This horse did not react to mallein prepared in the Imperial Bacteriological Laboratory at Muktesar, and when it was killed, four months later, no lesions could be found.

The Lister Institute stock strain of the glanders bacillus known as *B. mallei* A. also proved to be non-virulent. A guinea-pig inoculated subcutaneously with a hundred million organisms developed a small abscess, but it healed in a few days and when the guinea-pig was killed, four months later, there were no signs of disease.

The inoculation of a horse had a similar effect. A thousand million organisms from an agar culture, of *B. mallei* A., emulsified in salt-solution and injected beneath the skin of the neck, produced a local abscess which healed in three weeks and no lesions were found when the horse was killed four months later.

#### 6. THE SEROLOGICAL REACTIONS OF *B. WHITMORI* AND *B. MALLEI*.

When we began to investigate the agglutination reactions of melioidosis and to compare them with those of glanders we soon found that all our cultures of *B. whitmori* were serologically identical, but the five *B. mallei* strains were sharply divided into two groups or families, one of which is

clearly related to *B. whitmori*, while the other is more distantly connected with it. This conclusion was amply confirmed by the results of absorption tests and complement fixation.

The blood of the patient, Ragaviah, who was suffering from chronic melioidosis, agglutinated the Java and Muktesar strains *B. mallei* to the full titre of 1:4000, and the Minett strain to 10 per cent. of that amount. The Muktesar strain of *B. mallei* absorbed, from the blood of the patient, the agglutinins for the Whitmore bacillus cultivated from his own lesions, but the Minett strain did not. These reactions emphasise the relationship of *B. whitmori* to *B. mallei*; where it differs from *B. mallei* it always differs in a positive direction except that it is not pathogenic for horses. It is more robust and powerfully active than *B. mallei*, both within the body and outside it; it causes death more quickly in man and in laboratory animals; it grows more rapidly in artificial media; it forms a corrugated growth on glycerine agar; it takes only a few days to liquefy gelatine instead of several weeks, and, greatest difference of all, it is actively motile.

(a) *Agglutination reactions.* For the purpose of investigating the relationship of these organisms, we prepared ten immune sera by inoculating a series of rabbits with five strains of *B. whitmori* and five of *B. mallei*. The five strains of *B. whitmori* comprised three of human origin, namely, *Ragaviah* (corrugated), *Mananay* and *Warton*, and also two from animal sources, *Rabbit 45* (mucoid) and *Jacques's rat*. *B. mallei* sera were prepared with the five strains in our possession, viz. *Muktesar*, *Java*, *Minett*, *Egypt* and *A*. We have indicated the source of these cultures in the preceding pages of this paper. Each of the ten serums was put up in agglutination tests with emulsions of the fourteen strains of *B. whitmori* and the five strains of *B. mallei*, of which we have already given details. The results were as follows:

(1) Serum *B. whitmori*, *Ragaviah*.

Agglutinated all the strains of *B. whitmori* to full titre.

Agglutinated *B. mallei*, *Muktesar*, and *B. mallei*, *Java*, to full titre.

Did not agglutinate the following strains of *B. mallei*, viz. *Minett*, *Egypt* and *A*.

(2) Serum *B. whitmori*, *Mananay*.

Agglutinated all strains of *B. whitmori* to full titre.

Agglutinated *B. mallei*, *Muktesar*, and *B. mallei*, *Java*, to full titre.

Agglutinated *B. mallei*, *Minett*, *B. mallei*, *Egypt*, and *B. mallei A.*, to 3 per cent. of full titre.

(3) Serum *B. whitmori*, *Warton*.

Agglutinated all the strains of *B. whitmori* to full titre, except *Ragaviah* (corrugated) and *Mananay*, which it agglutinated to 50 per cent.

Agglutinated *B. mallei*, *Muktesar*, and *B. mallei*, *Java*, to full titre.

Agglutinated *B. mallei*, *Egypt*, and *B. mallei A.*, to 10 per cent.

Did not agglutinate *B. mallei*, *Minett*.

(4) Serum *B. whitmori*, *Rabbit 45* (mucoid).

Agglutinated all strains of *B. whitmori* to full titre, except *Mananay* and *Warton*, which it agglutinated to 25 per cent.

Agglutinated *B. mallei*, *Muktesar*, to full titre, and *B. mallei*, *Java*, to 25 per cent.

Did not agglutinate *B. mallei*, *Minett*, *B. mallei*, *Egypt*, or *B. mallei A.*

(5) Serum *B. whitmori*, Jacques's rat.

Agglutinated all strains of *B. whitmori* to full titre.

Agglutinated *B. mallei*, Muktesar, to 10 per cent.

Agglutinated *B. mallei*, Java, to 25 per cent.

Agglutinated *B. mallei*, Minett, to 2 per cent.

Agglutinated *B. mallei*, Egypt, and *B. mallei* A., to 10 per cent.

(6) Serum *B. mallei*, Muktesar.

Agglutinated all strains of *B. whitmori* between 10 and 20 per cent.

Agglutinated *B. mallei*, Java, to full titre.

Agglutinated *B. mallei*, Minett, *B. mallei*, Egypt, and *B. mallei* A., to about 2 per cent.

(7) Serum *B. mallei*, Java.

Did not agglutinate *B. whitmori* strains.

Agglutinated *B. mallei*, Muktesar, to full titre.

Did not agglutinate *B. mallei*, Minett, *B. mallei*, Egypt, or *B. mallei* A.

(8), (9) and (10). Sera, *B. mallei*, Minett, *B. mallei*, Egypt, and *B. mallei* A.

Did not agglutinate *B. whitmori* strains.

Did not agglutinate *B. mallei*, Muktesar, and *B. mallei*, Java.

Agglutinated to full titre, *B. mallei*, Minett, *B. mallei*, Egypt, and *B. mallei* A.

(These three sera were of comparatively low titre, about one in two thousand.)

Judged by the results of these agglutination tests the organisms fall into three groups: the first contains all the strains of *B. whitmori*, the second consists of *B. mallei*, Muktesar and Java, the third comprises the three strains of *B. mallei* from the National Collection of Type Cultures, namely, Minett, Egypt and A.

(b) *The absorption of agglutinins.* This method was employed to test the validity of the conclusions arrived at from a study of the agglutination reactions. First the five strains of *B. whitmori* which had been used in the production of immune sera were examined, and as they proved to be serologically identical only the strain known as *Ragaviah* (corrugated) was used in these experiments. Both *B. mallei*, Muktesar, and *B. mallei*, Java, were employed, and *B. mallei*, Minett, was selected to represent the third group. The results were as follows:

(1) Serum *B. whitmori*, Ragaviah.

Culture *B. mallei*, Minett, did not absorb the agglutinins from this serum.

„ *B. mallei*, Muktesar, absorbed all the agglutinins.

„ *B. mallei*, Java, absorbed 50 to 75 per cent.

(2) Serum *B. mallei*, Muktesar.

Culture *B. mallei*, Minett, did not absorb the agglutinins from this serum.

„ *B. mallei*, Java, absorbed 50 per cent.

„ *B. whitmori*, Ragaviah, absorbed all the agglutinins.

(3) Serum *B. mallei*, Java.

Culture *B. mallei*, Minett, did not absorb the agglutinins from this serum.

„ *B. mallei*, Muktesar, absorbed all the agglutinins.

„ *B. whitmori*, Ragaviah, absorbed all the agglutinins.

(4) Serum *B. mallei*, Minett.

Culture *B. mallei*, Muktesar, did not absorb the agglutinins from this serum.

„ *B. mallei*, Java, did not absorb the agglutinins.

„ *B. whitmori*, Ragaviah, did not absorb the agglutinins.

These absorption tests show that Whitmore's organism is serologically identical with the strain of the glanders bacillus which we obtained from Muktesar. Cross absorption between them is complete.

The Java strain of *B. mallei* differs from these two, because it contains a smaller number of antigenic components. Both *B. whitmori* and *B. mallei*, Muktesar, absorbed all the agglutinins from the Java serum; but the Java bacillus did not remove more than 75 per cent. of the agglutinins from either the *B. whitmori* or the Muktesar serum.

*B. mallei*, Minett, is only distantly related to *B. whitmori* and to the Muktesar and Java strains of *B. mallei*. A Minett immune serum agglutinated none of them, and none of them absorbed the agglutinins from it. *B. mallei*, Minett, removed none of the agglutinins from sera prepared with the Muktesar and Java strains of *B. mallei*, or from a serum prepared with *B. whitmori*. It was not agglutinated by any of these three sera to more than 10 per cent. of their full titre. There is, however, a group relationship between this organism and the other strains which is shown by the fact that the blood of the melioidosis patient, Ragaviah, agglutinated it to 10 per cent. of the full titre and these agglutinins were removed by saturation with the patient's own bacillus.

(c) *Fixation of complement*. Acting on the advice of Sir Arnold Theiler, whom we had consulted about melioidosis, we carried out complement fixation tests with the five following strains and their corresponding immune sera: *B. whitmori*, Ragaviah, and *B. mallei*, strains Muktesar, Java, Minett and A. The outcome of these reactions emphasises the close kinship of the *B. mallei* strains Muktesar and Java, with *B. whitmori*, and differentiates them quite clearly from the Lister Institute group Minett and A. The results are as follows:

(1) Serum *B. whitmori*, Ragaviah.

With antigen *B. whitmori*, Ragaviah, fixed 8 doses of complement (or more).

"	"	<i>B. mallei</i> , Muktesar,	"	5	"	"	"
"	"	<i>B. mallei</i> , Java,	"	5	"	"	"
"	"	<i>B. mallei</i> , Minett,	"	0	"	"	"
"	"	<i>B. mallei</i> A.	"	0	"	"	"

(2) Serum *B. mallei*, Muktesar.

With antigen *B. whitmori*, Ragaviah, fixed 8 doses of complement (or more).

"	"	<i>B. mallei</i> , Muktesar,	"	8	"	"	"
"	"	<i>B. mallei</i> , Java,	"	8	"	"	"
"	"	<i>B. mallei</i> , Minett,	"	0	"	"	"
"	"	<i>B. mallei</i> A.	"	0	"	"	"

(3) Serum *B. mallei*, Java.

With antigen *B. whitmori*, Ragaviah, fixed 8 doses of complement (or more).

"	"	<i>B. mallei</i> , Muktesar,	"	8	"	"	"
"	"	<i>B. mallei</i> , Java,	"	8	"	"	"
"	"	<i>B. mallei</i> , Minett,	"	0	"	"	"
"	"	<i>B. mallei</i> A.	"	0	"	"	"

(4) Serum *B. mallei*, Minett.

With antigen <i>B. whitmori</i> , Ragaviah, fixed 0 doses of complement							
"	"	<i>B. mallei</i> , Muktesar,	"	0	"	"	
"	"	<i>B. mallei</i> , Java,	"	0	"	"	
"	"	<i>B. mallei</i> , Minett,	"	8	"	"	(or more)
"	"	<i>B. mallei</i> A.	"	8	"	"	"

(5) Serum *B. mallei* A.

With antigen <i>B. whitmori</i> , Ragaviah, fixed 0 doses of complement							
"	"	<i>B. mallei</i> , Muktesar,	"	0	"	"	
"	"	<i>B. mallei</i> , Java,	"	0	"	"	
"	"	<i>B. mallei</i> , Minett,	"	6	"	"	
"	"	<i>B. mallei</i> A.	"	8	"	"	(or more).

The examination of the five strains of glanders bacilli from Java, Muktesar and the National Collection at the Lister Institute shows that the infecting agent in glanders is not always the same bacillus; a group of organisms, differing from each other in serological properties, has been included under the name *B. mallei*. Possibly the strains of the glanders bacillus which predominate among country-bred ponies in one part of the world, are not the same as those which are common in another, and these, again, may differ from the strains found in Arab horses.

*B. whitmori* differs from the strains of *B. mallei*, which we have examined, by reason of its growth on laboratory media and its active motility; but its immunity reactions show that it is closely related to certain of them and that serologically it is almost identical with the strain of *B. mallei* which we obtained from Muktesar. *B. whitmori* must, therefore, be regarded as a member of the glanders group which possesses certain peculiar physical attributes, namely, motility in young cultures and a corrugated appearance on glycerine agar.

## 7. SUMMARY.

(1) It is impossible to distinguish the lesions of melioidosis from those of glanders by means of inspection with the naked eye or even with the microscope.

(2) The symptoms and course of the two diseases are similar. Melioidosis generally runs a more acute course.

(3) The mallein test was applied in a case of chronic human melioidosis and gave a positive reaction. The mallein was obtained from Java.

(4) The blood of this chronic case agglutinated the Muktesar and Java strains of *B. mallei* to full titre and the Minett strain to 10 per cent. of this amount. One of these strains (Muktesar) absorbed the agglutinins for the homologous bacillus.

(5) There are very few horses in the Federated Malay States. Melioidosis is primarily a disease of rodents. All attempts to infect horses have been unsuccessful.

(6) Melioidosis broke out spontaneously among the rabbits, guinea-pigs and rats at the Institute for Medical Research in 1913. The animals became infected through eating contaminated food.

(7) Cases of infection in wild rats and in a domestic cat have occurred far away from this laboratory and independently of it.

(8) Rabbits, guinea-pigs and rats have been infected experimentally, by feeding, by subcutaneous inoculation, by scarification and by the application of infective material to the nasal mucosa.

(9) *B. whitmori*, whose growth in cultures occur in two forms, a commoner corrugated form and a mucoid form which gives origin to the corrugated type.

(10) *B. whitmori* differs from *B. mallei* in the following particulars. It is actively motile. It forms a corrugated growth on glycerine agar, a white opaque growth on ordinary agar and a pellicle on the surface of broth. It grows more rapidly than *B. mallei*, and it liquefies gelatine in a few days.

(11) *B. whitmori* resembles *B. mallei* in the following particulars. The morphology of the organisms is similar. Young cultures of the mucoid form are indistinguishable from *B. mallei*, by inspection. The growth of the mucoid form on potato is similar. The action on milk and carbohydrates differs in degree only. Both organisms produce Strauss's reaction in guinea-pigs.

(12) Five strains of *B. mallei* were compared with fourteen strains of *B. whitmori*. The cultures of *B. mallei* comprised three from the National Collection at the Lister Institute, one from Muktesar and one from Java. The cultures of *B. whitmori* had been isolated, some from human cases of melioidosis and some from animals which had acquired the disease naturally.

(13) The serological reactions of these organisms, namely agglutination, absorption and complement-fixation tests, showed that the cultures of *B. whitmori* were a homogeneous group, but the five strains of *B. mallei* were sharply divided into two sub-groups by their serological reactions. One sub-group includes the Muktesar and Java strains; the other comprises the three strains from the National Collection (Minett, Egypt and A.).

(14) The first sub-group of *B. mallei* is very closely related to *B. whitmori*. The serological reactions of the strain from Muktesar are almost identical with those of *B. whitmori*.

(15) The three strains of *B. mallei* from the National Collection, which form the second sub-group, are only distantly related to the Muktesar sub-group and to *B. whitmori*.

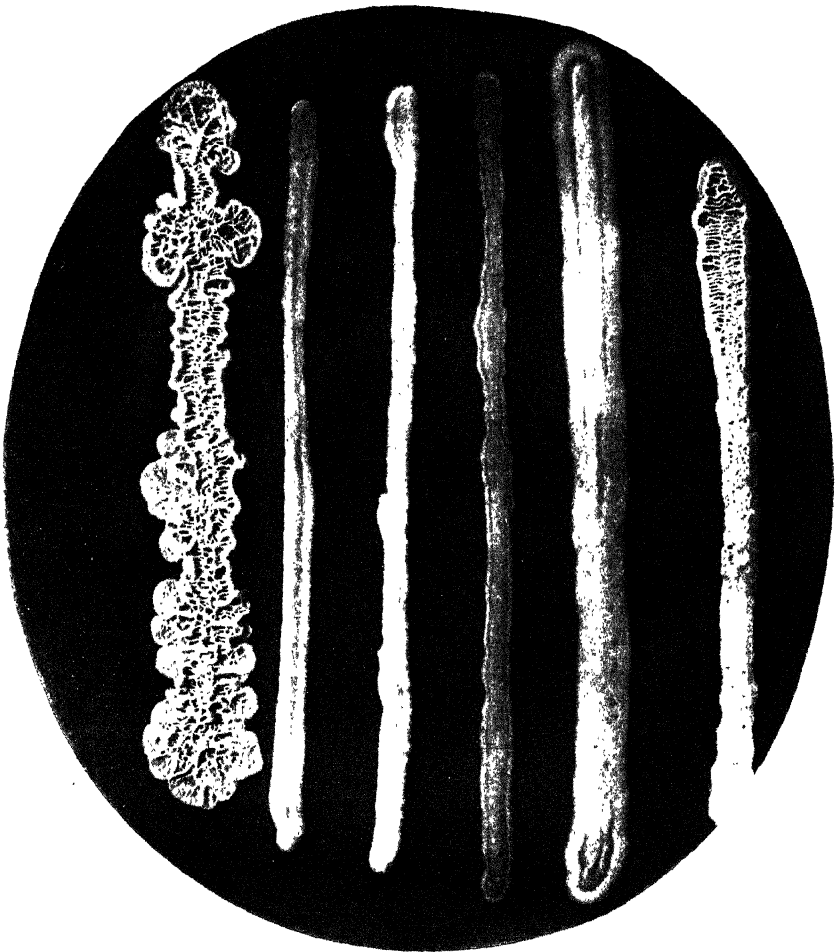
## 8. CONCLUSIONS.

*B. whitmori* is, serologically, almost identical with a strain of *B. mallei* from Muktesar, but it differs from it by reason of its motility and the character of its growth on laboratory media.

A strain of *B. mallei* obtained from Java is closely related to these organisms.

Three strains of *B. mallei*, from the National Collection of type cultures







at the Lister Institute, are distantly related to *B. whitmori* and to the strains of *B. mallei* from Muktesar and Java.

The causative organism of glanders is not always one and the same bacillus. A group of organisms differing from each other in serological properties has been included under the name *B. mallei*. *B. whitmori* is a member of this group, which has been found in rodents and in man.

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#### DESCRIPTION OF PLATE X.

*B. whitmori* and *B. mallei* on glycerine-agar. Seventy-two hours' growth.

Reading from left to right: *B. whitmori* (rat); *B. mallei* (A); *B. mallei* (Java); *B. mallei* (Muktesar); *B. whitmori* (mucoid); *B. whitmori* (cat).

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## A PRELIMINARY NOTE ON THE USE OF THERMOSTABLE OPSONINS (BACTERIOTROPINS) IN THE ELUCIDATION OF BACTERIAL INFECTIONS.

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ASSISTED BY MISS C. M. ACLAND.

(With 5 Charts.)

THERE are many forms of bacterial disease in which the search for the causal organism is complicated by the fact that it is present, not in pure culture, but associated with the number of other bacteria which may or may not be taking part in the infective process.

This is especially true of diseases of the respiratory and alimentary tracts, in which the search for the infecting germ necessitates the making of cultures from the sputum or the faeces. Under these circumstances, the usual serological tests, such as agglutinin estimations and the "complement fixation" technique are, for the most part, inapplicable, and the Bacteriologist is constrained to arrive at a provisional conclusion on such unsatisfactory grounds as the greater numerical preponderance of certain strains or the fact that some of the germs isolated are known to be more formidable than the others.

It is true that the estimation of the Opsonic Index, by the usual technique of Wright (1921) in which unheated serum is used, was at one time extensively applied for the above purpose, but this test is now seldom employed; nor is it entirely satisfactory, since it takes into account not only the specific but also the non-specific factors of phagocytic activity.

There remain the "thermo-stable" opsonins, the "Bacteriotropins" of Neufeld and Rimpau (1904), the "Incitor Elements," as they were provisionally called by Wright and Reid (1906), which are specific antibodies and which might, therefore, be expected to offer a better prospect of success.

How clear-cut and definite are the results of "thermostable" opsonic tests may be gathered from charts and tables published by the Author and Major C. C. Cumming illustrating investigations on *B. typhosus* and *B. paratyphosus* A (1912 and 1913) and upon the differentiation of *Staphylococci* (1913).

As to the specificity of the thermostable phagocytosis-inducing elements in serum there can be no doubt. This was demonstrated by Savtchenko (1902), by Neufeld and Rimpau (1904), and in this country by G. Dean (1905), as well as by many later observers. For a full bibliography of the subject, the article by F. Neufeld (1913) in Kolle and Wasserman's *Handbuch* may be

consulted. It has always been a source of surprise to the Author that these "bacteriotropins" have been so little used in the elucidation of problems like that now under consideration.

In the course of an extended investigation, still in progress, on the Tuberculo-Opsonic Index in patients suffering from phthisis, it became necessary to envisage the question of secondary infections in this disease; more especially as to the possibility of exploiting these "secondary" organisms in vaccine therapy with such guidance as might be had from a close investigation of the patient's serum.

It was thought necessary, however, to make as sure as possible that the germs used for making the "secondary" vaccines were really infecting the patients and not merely growing as saprophytes in the fluid contents of lung-cavities or upon the respiratory mucous membranes.

To this end, it was decided to compare the phagocytosis induced by both the heated and unheated serum of patients and healthy persons for each of the bacterial strains isolated. It was further decided to apply the same test to non-tubercular patients as occasion might arise.

Before drawing conclusions from this test as applied to human beings, it appeared desirable to ascertain the extent of the alterations in the relation of "bacteriotropins" to "opsonins" in the course of a specific immunization in animals; and the following experiment was accordingly carried out:

#### EXPERIMENT I.

Two rabbits (Nos. 16 and 17) were subjected to successive inoculations with a recent strain of *Staphylococcus aureus* of human origin, the injections being confined at first to heated emulsions given subcutaneously, and then passing on to the intraperitoneal inoculation of living cultures.

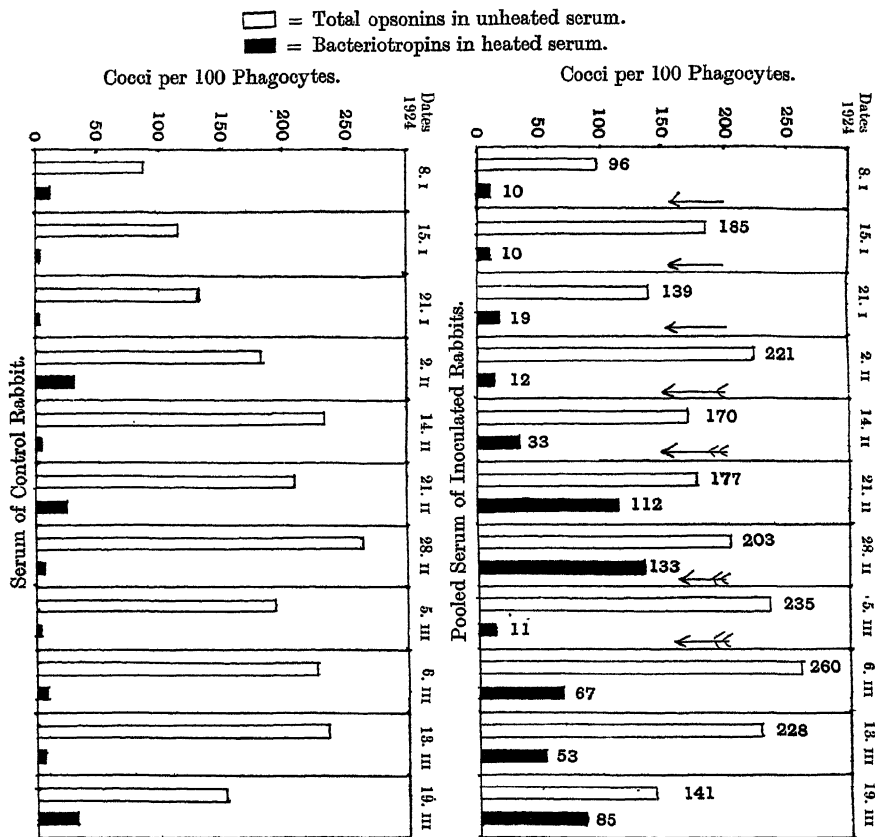
The serum of these two rabbits was collected at intervals and "pooled," the serum of a normal rabbit collected at the same time serving as a "control." Both sera were, for each examination, divided into two portions, one of which was inactivated by heating in a water-bath to 55° C. for half-an-hour, the other being unheated. Opsonic tests were now carried out by Wright's method with both the heated and unheated sera, a standardised living emulsion of staphylococci being used and human leucocytes being employed, always from the same source.

The results of this experiment are set forth in Chart A and serve to demonstrate that while the amount of opsonic power in the control serum is always greatly reduced by heating and remains insignificant throughout the experiment, the "bacteriotropins" in the serum of the inoculated group rise as the immunization proceeds while their quantitative relation to the opsonins steadily increases, attaining to over 65 per cent. when the process is at its height.

The marked fall in bacteriotropin observed on 5. iii. 1924 is to be noted.

On that occasion, the rabbits, through an error, were bled immediately after instead of, as usual, immediately before inoculation.

The apparent rise both in the opsonic power of the "test" and "control" serum may be due to the coccus having been frequently subcultured during the course of the experiment.



This Chart shows the gradual appearance of thermostable opsonins (bacteriotropins) in the course of the "immunization" of a group of two rabbits against *Staphylococcus aureus*. It will be seen that there is no formation of thermostable opsonins in the blood of a "control" rabbit of similar age and comparable weight.

- > Subcutaneous inoculation of heated culture of *Staphylococcus*.  
 -> Intraperitoneal " " "  
 >> " " living " "

Chart A (Experiment I.)

The fact that the opsonic power tended to be less in the "inoculated" than in the "control" group, although the bacteriotropins were rising in the former, is a matter of considerable interest. Here it is possible that physical factors were at work. The question of physical factors in the Opsonic Index is, however, too large for consideration here and is still under investigation.

The experiment, so far as it went, indicated that there was good ground for applying similar tests to human sera in the attempt to "incriminate" the infecting germs of a symbiosis from the merely saprophytic elements.

## EXPERIMENT II.

Ten patients suffering from pulmonary tuberculosis, for whom a "secondary" vaccine was desired, were investigated. From the sputum of each of these cases, two or more bacterial strains were isolated in pure culture and tested with the heated and unheated serum of the patient and a healthy "control" (C. M. A.).

The results are set forth in Table I.

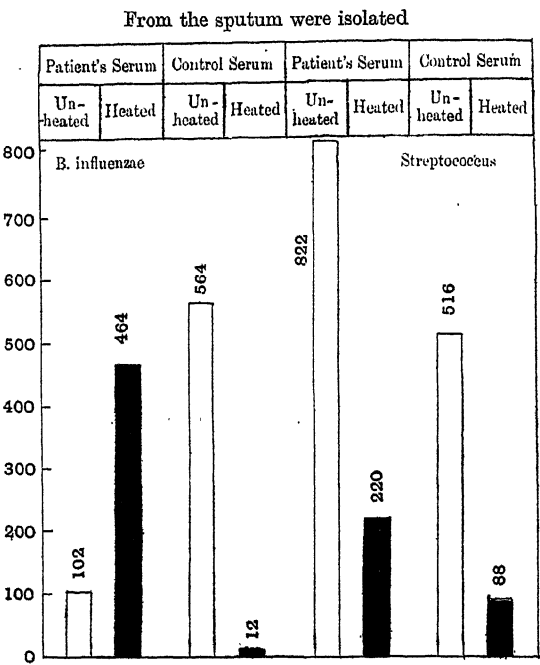
Table I.

*Bacteriotropin Tests in Phthisis Patients.*

Case. Name	Germ. Variety	Patient's serum Bacteria in 100 Phagocytes		Control serum Bacteria in 100 Phagocytes	
		Heated	Unheated	Heated	Unheated
<i>Positive Cases:</i>					
1. Miss F. W.	Streptococcus	108	122	30	184
	Pneumococcus	2	42	0	40
2. Miss L. D.	B. influenzae	464	102	12	564
	Streptococcus	220	822	88	516
3. M. J.	Streptococcus longus	156	144	44	156
	Streptococcus brevis	8	38	16	74
	? M. catarrhalis	46	268	80	260
4. V. W.	*Streptococcus non-haemolyticus	226	366	294	—
	Streptococcus hae-molyticus	798	570	326	—
<i>Doubtful Cases: (control insufficient)</i>					
5. R. W. J.	? M. catarrhalis	514	662	—	520
6. Mrs H.	Streptococcus	320	860	—	384
<i>Negative Cases:</i>					
7. Mrs D.	Streptococcus	12	294	4	346
	M. catarrhalis	14	102	34	244
8. Miss C.	Pneumococcus	2	120	78	216
	M. catarrhalis	8	462	82	388
9. J. E.	*Non-haemolyticus	102	342	116	370
	Streptococcus				
	M. catarrhalis	0	364	24	302
10. Miss E. E.	Staphylococcus	48	550	—	696
	Streptococcus	98	894	—	838

The words "Positive," "Doubtful," and "Negative" in this table refer to the results of the bacteriotropin test. All the patients were "positive" as regards the tubercle bacillus.

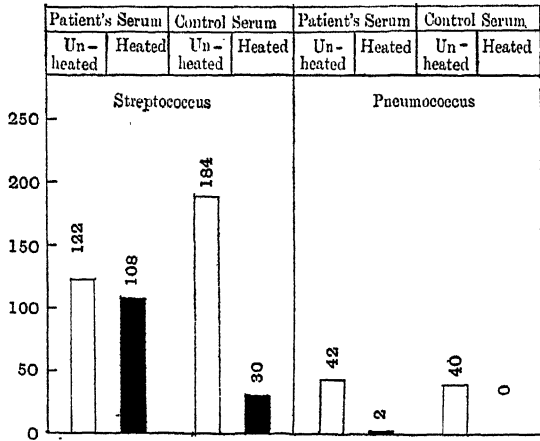
It is of interest to note that of nineteen "secondary" organisms isolated from the sputum of ten phthisical patients, there was definite evidence to incriminate seven organisms as actually infecting the tissues; while it is probable that two other strains, making a total of nine, were etiologically connected with the patient's condition. In the case of the two doubtful



The high thermostable opsonin for *B. influenzae*, which contrasts sharply with the control, indicates specific infection with the germ. There is reason to think that the streptococcus, too, is infecting.

Chart B. (Experiment II. Case 2. Table I.)

Streptococcus and Pneumococcus isolated from sputum.



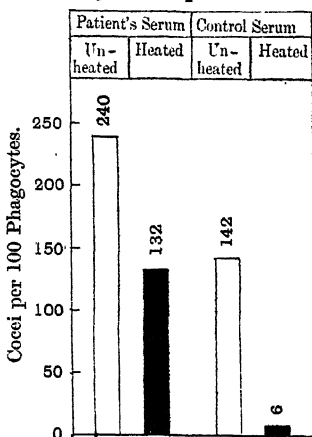
This test indicates that there is specific infection by the streptococcus, for which the thermostable opsonins represent 88 % of the total; while it is clear that the Pneumococcus is merely a saprophytic element.

Chart C. (Experiment II. Case 1. Table I.)



organisms, marked with a "star" in the table, it will be noticed that both were non-haemolytic streptococci and that, in both instances, the doubt arose

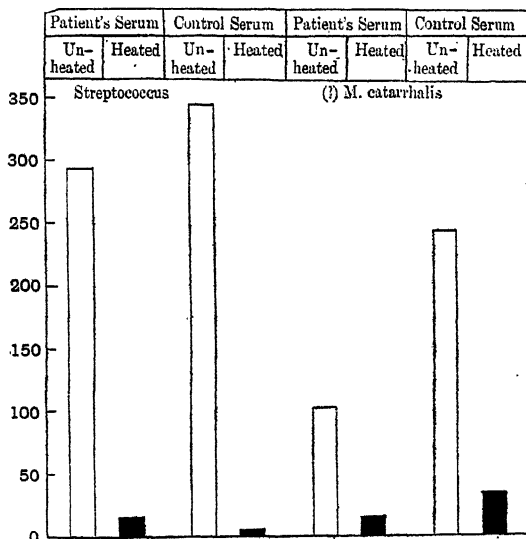
Osteomyelitis Staph. aureus.



Here there is definite evidence of specific reaction to infection, the patient's thermostable opsonins being 55 % of the total; the control being just over 4 % only.

Chart D. (Experiment III. Case 1. Table II.)

The organisms cultivated from the sputum were a streptococcus and (?) *M. catarrhalis*.



In this patient, there is no evidence to suggest that either of the germs isolated has infected the tissues. Both organisms would appear to be purely saprophytic.

Chart E. (Experiment II. Case 7. Table I.)

owing to the presence of equally high or higher bacteriotropins in the heated serum of the "control."

It is probable that this finding should be interpreted, not as exempting

the germ as an agent of infection of the patient but rather as incriminating it as infecting the "control" also.

The sharp differences elicited in this test are more easily appreciated graphically; and three "positive" tests are illustrated in Charts B, C and D, a "negative" result in Chart E.

Chart B (from Case 2) is especially interesting in that the opsonins for *B. influenzae* in the unheated serum appear as in the case of the immune rabbits in Exp. I to be less in amount than those in the heated serum. It will be noticed that a similar phenomenon occurs with a *Streptococcus longus* in Case 3 and, more markedly, with a *Streptococcus haemolyticus* in Case 4.

The authors (Cummins and Acland, 1923) have sometimes found this to happen with tubercle bacilli, especially in advanced cases, and have also reproduced the same finding in rabbits infected with Koch's bacillus. The phenomenon may perhaps depend on an increase of "antibody" above the optimum concentration for the strength of antigen used, the heating of the serum reducing the total opsonin to a more favourable concentration and thus leading to increased phagocytosis. H. R. Dean (1911) has shown, in Complement Deviation tests with bacteria, that such a quantitative relationship does actually exist between antigen and antibody, excess of either constituent being unfavourable to the fixation of complement.

### EXPERIMENT III.

The bacteriotropin test was also applied in a group of ten non-phthysical patients, details of these cases being given in Table II.

It will be noticed that, in this group of cases, the evidence to be gained from the test is less sharp, as a rule, than in the phthysical group where existing lesions may be supposed to have given a readier access of the secondary germs to the tissues.

Case 11, a patient suffering from osteomyelitis, is perhaps the most striking. Here the answer was definitely positive.

Cases 12 and 15 are of interest, both being instances of acute streptococcus empyema following influenzal attacks. In both, the organism was isolated in pure and profuse culture from the pus aspirated by means of a syringe. It may be assumed, therefore, that the streptococci were from the respiratory tract and not from the skin. In Case 15, the test may be taken as indicating that there had not, as yet, been much general infection by the streptococcus isolated and which was pullulating in the liquid contents of a closed serous sac. That some infection was taking place was, however, strongly suggested by a "dilution opsonic," carried out at the same time but not here illustrated; and the result of the bacteriotropin test must therefore be noted as a mark *against* its value in this instance. In Case 12, the emulsion used was very much too dilute, as the result of difficulty with "clumps" which had to be got rid of by centrifugalisation. This explains the negative findings in both

heated and unheated "control" serum. The amount of bacteriotropin in the patient's heated serum, though higher than in the "control," was very low in proportion to the opsonic power of the same serum unheated; but the observation clearly indicates that the germ was "infecting," the most striking result being that obtained with the patient's unheated serum, more especially in consideration of the very dilute bacterial emulsion used.

Table II.

*Bacteriotropin Tests in Non-phthisical Patients.*

Case.	Name and disease	Germ. Variety	Patient's serum Bacteria in 100 Phagocytes		Control serum Bacteria in 100 Phagocytes	
			Heated	Unheated	Heated	Unheated
11.	Mrs S. Osteomyelitis (pus)	Staphylococcus aureus	132	240	6	142
12.	MacA. Empyema (pus)	Streptococcus	14	322	0	2
13.	Miss M. G. Lupus facialis (culture from lesion)	Streptococcus	30	530	24	104
		Staphylococcus aureus	102	822	160	198
14.	J. S. C. Colitis (faeces)	B. coli Streptococcus	24 10	12 118	14 22	40 52
15.	A. E. F. Empyema (pus)	*Streptococcus	76	506	124	508
16.	C. Robinson Chronic bronchitis (sputum)	*Streptococcus	256	632	260	—
		? M. catarrhalis	392	402	62	—
17.	S. L. C. Chronic cold (sputum)	M. tetragenus *Streptococcus viridans	4 204	40 398	14 336	72 554
18.	S. L. C. Rheumatism (faeces culture)	Streptococcus faecalis	20	434	4	146
		Gram + bacillus *B. coli	4 14	46 190	— 40	72 62
19.	Mrs S. Colitis (faeces culture)	Staphylococcus	8	150	—	474
		*B. coli Gram + bacillus	216 —	700 230	156 2	164 232
20.	K. M. H. Chronic cold (sputum)	*Streptococcus	234	674	258	284
		*? M. catarrhalis ? M. pharyngeus	146 100	678 400	672 52	564 208

Case 13, too, is of interest as showing that there are occasions when the comparison of the unheated sera of patient and control gives a clear answer when the bacteriotropin test is negative. It will be remarked that the strains tested in this case were from the surface of an extensive and long-standing lupus patch, under which, no doubt, the lymphatic drainage was almost completely obliterated by the tuberculous process. Under these circumstances, the auto-inoculations of staphylococcus and streptococcus must have been considerably reduced.

Cases 12, 13 and 15 suggest that where the infective agent has only very recently come into operation, as in acute cases, or where, for some mechanical reason, an old-standing infection is almost completely shut off from the circulation, the bacteriotropin test is liable to fail. In such cases the standard

"opsonic index" readings, according to Wright's method, would appear more likely to be of service.

In Case 14, the observation as regards the streptococcus proved that this was "non-infecting" and, in the case of the *B. coli*, though suggesting the presence of bacteriotropins, the test was unsatisfactory through a technical error in using too thin an emulsion. A vaccine of the colon bacillus and streptococcus gave excellent results.

In Case 16, the test was satisfactory and there seems definite evidence to incriminate the *M. catarrhalis*.

Case 17 requires further comment.

In the course of an acute exacerbation of a chronic cold met with in one of us (S.L.C.) it was found that while there were no specific antibodies for a strain of *M. tetragenus* from the throat, there were high thermostable opsonins for a *Streptococcus viridans* also present in large numbers on the plates. It was found, however, that the thermostable opsonins for the latter organisms were even higher in the control serum (C. M. A.).

It happened that the donor of the control serum in this instance had been acting for months as Assistant to the "Patient" and had also, during this period, suffered from several "colds." A swab taken from the throat of the "control" the day after the test produced numerous colonies of *Streptococcus viridans* on plating. In Case 20, the bacteriotropins and opsonins for each of the three organisms tested were present in about the same degree, except in the case of *M. catarrhalis* in which the "control" serum was very rich and no definite conclusions could be drawn. Here, too, the "patient" and "control" had for long been exposed to the same source of catarrhal infection.

These findings raise once more the question of the occasional presence of a considerable measure of bacteriotropins in the serum of healthy persons.

I have marked with a star the seven organisms out of the twenty bacterial strains dealt with in Table II for which the "control" (C. M. A.) possessed bacteriotropins, and it will be noticed that, as in the case of the organisms similarly "starred" in Table I, four of these germs are streptococci.

The isolation of *Streptococcus viridans* in large numbers in throat swabs from this "control" gives point to the above observations. It is now too late to be sure whether the other streptococci for which bacteriotropins were demonstrated in the control (C. M. A.) serum were of the "viridans" type, as the time available did not permit of satisfactory classification of all the strains isolated; but it is at least known that none of them were haemolytic.

It has long been recognised that "normal" sera contain a variable amount of thermostable opsonin, especially for the more widely distributed organisms such as tubercle bacilli, colon bacilli and staphylococci. It seems probable that this depends on occasional infection or sub-infection by the organisms concerned or their near antigenic relatives. In the case of such a widely distributed and persistently infective organism as the tubercle bacillus, this fact amounts to a serious interference with the value of the bacteriotropin test;

but for less resistant or less common bacteria it is not difficult to find suitable "controls."

From the point of view of technique, the method used was that devised by Sir A. E. Wright for the estimation of the Opsonic Index. Films were made with the type of "spreader" introduced by him; the staining was carried out with Leishman's stain; and no account was taken of the content of any cellular type except the polymorphonuclear phagocyte. Except in two unavoidable instances, all the enumerations were carried out by the same observer (S. L. C.). Control serum and washed blood cells were always from the same source (C. M. A.).

In Exp. I, the opacity of the bacterial emulsion was always adjusted to correspond to No. 1 on Brown's scale (1914). In Exps. II and III, the emulsions were "guessed" to about a suitable opacity but were not standardised by comparison with the scale. It was an invariable rule that the Enumerator remained in ignorance of what film he was counting, the slides being marked by an Assistant, "shuffled" and dealt to him in any order that chance happened to dictate. It was only when the day's counting was completed that the "results" were "assembled" and sorted into their proper order. There was, therefore, no question of unconscious bias.

As was to be expected in a group of advanced phthisis cases, the "secondary vaccine" treatment based on the above tests gave but inconclusive results. One point of interest, however, emerged; as it was found that there was definite "sensitivity" to the vaccines used in Cases 2, 3 and 5, the patients exhibiting a tendency to marked reactions after even minimal doses.

There were no reactions of any kind amongst the "negative" cases to any of the mixed vaccines made for them from their own germs. This bacterial sensitivity of infected persons, noticed, too, in several of the non-phthisical cases, requires to be taken into account in determining the doses of vaccines, especially in the case of debilitated patients.

Amongst the non-phthisical patients, marked benefit followed vaccine treatment in Cases 11, 12, 14, 17 and 20. There was no benefit in Case 13. In Case 15, no vaccine was used, and in Case 16, the patient, a West Indian negro, left the hospital too soon to allow evaluation of the treatment. In Case 18, there was marked sensitivity to the vaccine of streptococcus and *B. coli*. In Case 19, the results are not yet to hand as the patient is travelling abroad.

It is hardly to be expected that a laboratory test involving a considerable amount of time should come into general use in the guidance of vaccine therapy; but, in intricate cases where there appears to be special reason for caution in dosage, the bacteriotropin test would appear to be well worth the trouble.

## CONCLUSIONS.

1. The comparison of the bacteriotropins and opsonins of the patient's serum is often of definite value in distinguishing between the infective agents and the harmless saprophytes in the bacterial flora of sputum, faeces, etc.

2. The fact that "healthy" control serum may contain well-marked bacteriotropins for certain bacteria must be taken into account in interpreting the findings and may necessitate the use of several "controls" as suggested by Wright.

3. The presence of bacteriotropins in the serum of a patient should be taken as suggesting that there may be marked sensitivity to the germ in question. In such cases, the greatest caution in dosage is necessary.

4. From the point of view of clinical medicine, it is of interest to note that there was evidence of secondary infection by nine out of nineteen bacterial strains, other than tubercle bacilli, isolated from the sputum.

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## ON STAPHYLOCOCCAL PRECIPITIN REACTIONS IN CASES OF ACUTE AND CHRONIC INFECTIONS AND ALSO IN SERUM SICKNESS.

By

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### INTRODUCTION.

THIS work was undertaken for the purpose of determining the occurrence and specificity of bacterial precipitins in infections caused by the *Staphylococcus aureus*. The differences in antigenic properties of various strains of orange and white staphylococci employed in the preparation of the filtered antigens and the importance of the presence of peptone in this connection led to numerous experimental observations which had not been anticipated. The presence of strong *S. aureus* precipitins in the blood of an adult suffering from very acute serum sickness following an injection of anti-diphtheritic serum led to an investigation of the blood of patients suffering from serum sickness, the results of which are fully recorded here. The investigation of the blood of normal rabbits revealed the fact that in some of these animals staphylococcal precipitins were present, which led to unexpected difficulties in the study of precipitins in the actively immunised animal.

### TECHNIQUE.

*The Patients' Blood.* Blood was withdrawn always by vein puncture into sterile test tubes, which were slanted until the serum had separated. The serum was centrifugalised until it was quite clear or showed only turbidity due to fats or lipid substance. It was then pipetted off into sterile test tubes and heated in a water bath at 58° C. for twenty minutes, and tested with the antigens within twelve hours of obtaining the samples of blood. This was the routine procedure, but comparative tests were made on numerous occasions with unheated sera and with sera which had been heated at 55° C. for thirty minutes, and 60° C. for ten minutes.

*Rabbits' Blood.* Test samples were obtained from a vein of the ear in the usual way and the blood serum was prepared by the same technique as described for human blood. Rabbits which were immunised were bled from the axillary artery and the serum when separated was stored in sterile bottles

undiluted, or diluted with saline. Preservatives such as 0.1 per cent. formalin, or 0.25 per cent. phenol were employed.

*The Reaction.* Human or rabbit's serum was diluted with fresh sterile saline containing 0.25 per cent. phenol in all these experiments, and the dilutions employed were 1 in 5, 10, 20, 40, 80, 160 and occasionally 1 in 320. We used 1 c.c. of the diluted serum and 0.5 c.c. of one of the clear antigens<sup>1</sup>; control experiments were made by reversing the proportions of antigen and anti-sera, but the former was the routine procedure. At the outset of the work the mixture of antigen and diluted serum was placed in glass tubes ( $3\frac{1}{2} \times 3/10$ ) in a closed water bath. We employed a constant temperature of 37° C. for 48 hours, taking readings at 24 and 48 hours respectively against a black background, but the long incubation at 37° C. in spite of the presence of the small quantity of antiseptic, allowed bacterial contamination in some instances. We, therefore, after full experimental observations abandoned the temperature of 37° C. and employed open water baths at 52° C. for 48 hours entirely for these experiments, although Kraus (1897), Nicolle (1898) and Radziewsky (1900) all found that a temperature of 37° C. was most satisfactory for bacterio-precipitins. The following Table shows the method we considered to be the most satisfactory for these experiments.

Table I.

Antigen (undiluted) (c.c.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	—	—	0.5
Saline (c.c.)	—	—	—	—	—	—	—	0.5	0.5	1.0
Serum (c.c.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	—
Dilution of serum <sup>1</sup>	1 in 5	10	20	40	80	160	320	5	20	—

The precipitate present was recorded in the following manner and is referred to by these abbreviations: T or trace, a granularity remaining in suspension, + a distinct granularity with a deposit, ++ a deposit of much greater intensity so as to completely cover the convex surface of the tube or give a milky turbidity, and finally +++, a still heavier deposit without granularity of the supernatant fluid. 0, signifies no reaction and — that the reaction was not made. The deposit might be granular like very fine sand, or definitely flocculent. Both varieties occurred in reactions of equal intensity, and as a whole the flocculent was more common with immune rabbit sera than with the human, but both varieties of precipitate occurred with normal and immune rabbit sera, and with human sera obtained from cases of serum sickness or *S. aureus* infections.

The deposit from the positive tubes examined microscopically was found to consist of a granular débris.

*Control Reactions.* Control observations were made with 1 per cent. peptone in normal saline, horse serum, anti-toxic horse serum, beef broth, a 1 per cent. solution of starch. All the reactions were carried out by the routine method already referred to in detail and by various modifications.

<sup>1</sup> In the total volumes the dilutions of serum are therefore 1 in 7.5, 15, 30, 60, etc.



*Preparations of Antigens.* The various antigens used, except where specially stated to the contrary, consisted of a basis of beef broth to which 1 per cent. of peptone and 0·5 per cent. of sodium chloride were added, and gave a reaction of 7·5 to the universal indicator. Each flask which contained 200 c.c. of the medium was autoclaved at 110° C. for twenty minutes. The peptone employed in the majority of these experiments was obtained from Messrs Allen and Hanbury and is known as "blood peptone," but other samples of peptone obtained from Hopkins and Williams, May and Baker, and the peptone sèche de Chapoteaut appeared to be of equal merit, and the experiments referred to in the section on serum sickness indicate that this belief is correct and that any of the four samples of peptone referred to may be employed. Antigens were also made of horse flesh instead of beef, marmite, and beef broth without the addition of peptone. A limited number of comparative experiments were made with cultures of the *S. aureus* grown in the broth flasks at 37° C. for one week, two weeks and three weeks, but apart from these observations, all our experiments were made with antigens which had been incubated at 37° C. for one month, then filtered through carefully washed Berkefeld candles (of pre-war German make) by the aid of a water exhaustion pump, and to which 0·1 per cent. formalin was added. The sterile filtrates were stored in bottles with paraffined corks in the dark at room temperature, and the necessary amount was readily withdrawn when required. By this method of filtering and storing, absolutely bright and clear antigens were always obtained and were seldom contaminated. We also tried 0·25 per cent. phenol, and toluol, as preservatives, but 0·1 per cent. formalin was found to be the most satisfactory agent. Graham Smith and Sanger (1903) also found that in dilutions below 1 in 100 the addition of formalin did not interfere with serum precipitation.

The flasks of peptone beef broth were inoculated with strains of *S. aureus* obtained from pus, sputum, and by blood culture, but the strain most commonly employed as it always gave an efficient antigen is referred to throughout this communication as "Orpen." This culture of *S. aureus* was isolated from a purulent sample of sputum from a case of bronchitis, but antigens prepared from other strains, however, of *S. aureus* were equally efficient. Antigens were also prepared from cultures of the *S. albus* obtained from a bone abscess, pustular acne, and human milk, from haemolytic and non-haemolytic strains of *Bacillus coli*, from *B. proteus*, *B. typhosus*, *B. paratyphosus* A, B and C, and *B. diphtheriae*.

#### CASES OF *STAPHYLOCOCCUS AUREUS* INFECTION, AND CONTROL CASES.

Blood sera were examined for the presence of *S. aureus* precipitins in 118 cases of *S. aureus* infection. In all these cases the organism had been isolated from the blood or from the pus, sputum, or other exudates. It was obtained in pure culture in all the cases of furunculosis, carbuncle, osteomyelitis, and septicaemia, and in almost all the cases of abscess formation. In some of the

remaining cases such as those of pulmonary, uterine, and urinary infections other organisms were present, but the number of staphylococci was so great as to indicate that this organism had played an important part in the causation of the infection. The results of these investigations are shown in the following table (II).

Table II.

	Total cases examined	Total negative	Total positive	Positive			
				Very strong (+ + +)	Strong (+ +)	Moderate (+)	Weak (Trace)
Furunculosis	66	34	32	4	5	13	10
Carbuncle	7	2	5	1	0	1	3
Abscess	19	8	11	2	1	5	3
Osteomyelitis	5	3	2	0	1	0	1
Cellulitis	5	1	4	1	0	1	2
Septicaemia	2	1	1	0	0	0	1
Other infections	14	4	10	5	2	1	2
Total	118	53	65	13	9	21	22

A very large number of sera were tested against two, three or more filtrates made from different strains of *S. aureus*, because sera which gave a strong reaction with one filtered antigen might give a weak reaction with another or fail to react. A general review of all the sera tested showed that of the antigens employed the "Orpen" and the "Nauen" (from a case of *S. aureus* pyaemia) antigens gave the best results in most cases. This, however, was not invariably the case, and in a few instances other antigens gave better results than either of the above, and further some sera showed a marked difference in the strengths of the reactions obtained with the "Orpen" and "Nauen" antigens.

In determining the strength of the precipitin reactions it was necessary to make allowance in those cases which, as is so often seen in agglutination experiments, showed zonular reactions, which are referred to by Kolmer. We have noted that many sera which gave zonular reactions at the end of 24 hours' incubation have subsequently undergone marked changes, so that at the end of 48 hours the zones had disappeared. Many zonular reactions can also be avoided if the sera are tested on the same day on which the blood is collected. It has been stated previously that the sera were heated before use at 58° C. for 20 minutes, and it was thought that this procedure might have some tendency towards the production of an inhibition zone, but comparative experiments made with heated and unheated sera showed that there was no appreciable difference. Further, it was noted that whereas a zone might be obtained with an antigen made from one strain of *S. aureus*, with antigens made from other strains no such zone was apparent. This variation in the reaction with antigens made from different strains of *S. aureus* was not infrequent and since, as stated above, many of the sera were tested with two or three different antigens, it was possible to obtain a more accurate estimate of the amount of the precipitate formed with the lower dilutions of the sera.

In a certain number of cases also the sera were examined for precipitins

with antigens prepared from other organisms, e.g. *B. typhosus*, *B. paratyphosus* B, *B. coli* (two or three strains). In one or two cases the presence of a co-existing infection was definitely established and in these cases co-existing precipitins were demonstrated in the sera. The following example may be given: Miss S. suffering from typhoid fever (positive blood culture). Towards the end of the third week septic foci, from which *S. aureus* was recovered, appeared in various parts of the body and numerous colonies of *S. aureus* were isolated from the faeces. This condition persisted for over a fortnight. An examination of the patient's serum showed the following results:

	1/5	1/10	1/20	1/40	1/80	1/160
$\bar{c}$ . <i>S. aureus</i> (Nauen) filtered antigen	++	+	T*	T	T	0
$\bar{c}$ . <i>B. typhosus</i> filtered antigen	+	++	++	++	++	T

\* T = Trace.

In other cases where there has been no evidence of co-existing infection no reaction has been obtained with antigens other than those prepared from *S. aureus*.

In a number of cases the same sera were tested against antigens prepared from *S. albus* of which four strains were employed. One of these strains was isolated in pure culture from a case of acute infective osteomyelitis and differed from most cultures of *S. albus* in fermenting mannite. Sera which showed positive reactions with *S. aureus* antigens also reacted with this antigen, but no reactions were obtained with *S. albus* antigens prepared from the remaining three strains, which were isolated from two cases of pustular acne and from normal human milk.

It will be seen from Table II that out of 118 sera examined in cases of *S. aureus* infection 53 or 45 per cent. were completely negative; 65 or 55 per cent. were positive, but of these 22 or 18 per cent. showed only weak reactions. Of the 66 cases of furunculosis, many of which were chronic cases with several recurrences, 34 or slightly over 50 per cent. showed no precipitins for *S. aureus*. Nor did we find any evidence that the precipitin content was necessarily increased as the result of vaccine treatment. A number of cases of furunculosis were examined both before and after a course of injections with *S. aureus* vaccine, but apart from two cases which showed a slightly increased reaction no change occurred.

In addition to the preceding a series of 98 sera were examined from cases in which there was no *à priori* reason to suspect the presence of an infection necessarily due to *S. aureus* and this organism was not isolated from any of the patients. The majority of the individuals who furnished these sera were in good health and considered normal. Of these 98 sera 56 or almost 58 per cent. were completely negative, nine showed a very strong reaction, twelve a strong, eleven a moderate and ten a weak. It is impossible however from an examination of the past history to exclude definitely the previous existence of infection caused by *S. aureus*. Further, we wish to draw attention to the frequent presence of *S. aureus* in the intestinal flora. The faeces were examined in

17 cases chosen at random from a medical ward in the hospital, cases in which there was no reason to believe that a staphylococcal infection existed, but in four of these cases colonies of *S. aureus* were obtained. Of the cases in which there is a definite *S. aureus* infection the proportion showing the presence of this organism in the faeces is much greater. It is suggested that in some cases where no other cause can be ascertained the presence of precipitins in the serum might be associated with this infection of the intestinal tract. In addition, as will be noted later, a history of previous serum treatment is of the utmost importance. The following case may be quoted: O.W., complaining of no symptoms and apparently healthy. In April, 1915, he received anti-tetanic serum without reaction. In April, 1916, he received several further doses of anti-tetanic serum. A very marked serum sickness followed with severe urticaria, vomiting, and oedema. His serum tested in November, 1923, showed a very strong precipitin reaction with *S. aureus* (Orpen) filtered antigen. A perusal of the results obtained in cases of serum sickness and given in the subsequent section of this paper shows however that the persistence of this reaction for such a long period of time is unusual.

It would appear also that cases of asthma require special consideration. Walker (1916) has found that the proteins of bacteria commonly found in the sputum of asthmatics may be the primary exciting agents and notably of *S. aureus*. We have examined the sera from six cases of asthma. One which showed a large number of colonies of *S. aureus* in culture from the sputum gave a strong precipitin reaction. Of the remaining five, from none of which *S. aureus* was recovered, one gave a strong, one a moderate, one a weak, and two a completely negative reaction.

Whether precipitins for *S. aureus* are found in the blood of some normal individuals, as in some rabbits, is difficult to determine with certainty, but it is possible that this may be the case. The percentage of sera, from healthy normal individuals and from patients suffering from affections unassociated with *S. aureus*, which give positive precipitin reactions is large. The impossibility, however, of definitely excluding a previous *S. aureus* infection and the importance of other considerations mentioned above render the difficulty apparent.

#### THE EXAMINATION OF THE BLOOD IN CASES OF SERUM SICKNESS WITH FILTERED *STAPHYLOCOCCUS AUREUS* ANTIGENS.

The venous blood of patients who had received serum treatment was examined for the presence of precipitins with filtered cultures of *S. aureus* grown for one month at 37° C. in peptone beef broth. The details of the preparation of these antigens and of the technique employed has been already described. It may be necessary, however, to emphasise that these reactions were carried out in a water bath at a temperature of 52° C. and readings were taken at intervals of 24 and 48 hours. Comparisons were also made at a temperature of 37° C. and with other antigens to be referred to. The majority

of these patients had been treated with anti-diphtheritic serum, but a few had received anti-tetanic, anti-meningococcic, or anti-streptococcic serum, but each anti-serum was prepared from horses. Within 24 hours of collecting the blood the sera were tested with one or more of the various antigens. In all, 109 cases were investigated, and as already stated the majority of the patients had received anti-diphtheritic horse serum in doses varying from 2000 to 73,000 units subcutaneously. The blood was collected in most instances in direct relation to the appearance of the rash and other manifestations of serum sickness, but occasions occurred when the blood was collected many days after the rash had faded, and in a few instances within 24 hours before the rash appeared. Some patients whose blood gave a strong reaction were re-examined after convalescence had been established, and it was then found that a negative or only very weak reaction remained. It is interesting to note that Wells (1915) found precipitins for horse serum in the patient's blood occasionally before the onset of serum sickness. He found that these precipitins were present in low concentration during serum sickness, but that the strength of the reaction increased rapidly towards the end of the illness.

In the total of 109 cases, 43 very strong positive reactions, 21 strong, and 22 moderate occurred, in 9 instances a weak reaction and in 14 a negative was recorded. These results give a percentage total of 87·3 per cent. positive of which 79·6 per cent. gave a well-marked reaction. In eight cases which had received anti-diphtheritic serum a positive reaction was obtained and in five of these cases it was well marked, but there was complete absence of clinical evidence of serum sickness. Wyard (1912) made observations on serum precipitation on 51 men who had received one or more injections of horse serum. He was unable to establish any connection between serum sickness and circulating precipitin for horse serum in the patient, as those whose blood contained these antibodies were in no way more likely to suffer from serum sickness than those who were free from them. The blood of 30 patients who gave a positive reaction of varying intensity with *S. aureus* antigens failed to react with *S. aureus* antigens prepared *without the addition of peptone to the medium*; and with *S. albus* antigens made with peptone. In addition many sera from cases of serum sickness which gave marked reactions with *S. aureus* antigens gave completely negative results when tested with antigens similarly prepared from *B. typhosus*, *B. paratyphosus* B, *B. coli*, *B. proteus*, and with diphtheritic toxin—a large number of these sera were also tested with 1 per cent. peptone solution under identical conditions, but negative results were obtained.

In Table III is shown the results of the blood examination of four cases of serum sickness with four beef broth antigens of *S. aureus* (Orpen) prepared from four distinct preparations of peptone.

These experiments are of special interest as they show the effect of preparing the beef broth antigens with four different samples of peptone. The same strain of *S. aureus* (Orpen) was used to inoculate each flask and the

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flasks were then incubated at 37° C. for one month. The blood was collected from four patients suffering from serum sickness and each experiment was carried out with the same four antigens and the same technique was employed. The results obtained in each experiment are almost identical, which shows that this strain of *S. aureus* is able to make use of the four samples of peptone to the same extent. Since this observation was made our findings have been confirmed with further samples of sera.

Table III.

No.	Age	Amount of anti-diphtheritic serum injected units	Interval between injection of anti-serum and appearance of rash days	Interval between injection of anti-serum and blood examination days	Result of Blood Examination (Dilution of patient's serum)						Antigen employed. <i>Staphylococcus aureus</i> (Orpen)
					1 in 5	10	20	40	80	160	
1	4½	54,000	13	14	+	+	+	+	T*	T	1. Beef broth. A. and H.'s blood peptone
					+	+	+	+	T	T	2. Beef broth. Hopkins and Williams' peptone
					+	+	+	+	+	T	3. Beef broth. May and Baker's peptone
					+	+	+	+	T	T	4. Beef broth peptone. Sèche de Chapoteaut
2	4½	9,000	9	10	+	+	+	+	T	T	Antigens 1-4 employed in same order as above
					+	+	+	+	T	T	
					+	+	+	T	T	T	
					+	+	+	T	T	T	
3	11	9,000	15	16	T	T	T	0	0	0	Antigens 1-4 employed in same order as above
					+	T	T	0	0	0	
					+	T	T	0	0	0	
					+	+	T	0	0	0	
4	21	12,000	9	10	+	T	T	T	T	0	Antigens 1-4 employed in same order as above
					+	T	T	0	0	0	
					T	+	T	0	0	0	
					T	T	T	0	0	0	

\* T=Trace.

The blood reactions of six cases of serum sickness were compared as regards our *S. aureus* precipitin reaction and precipitation with antitetanic horse serum. For this reaction 0.5 c.c. of serum obtained from cases of serum sickness was added to each tube of 0.5 c.c. of anti-tetanic horse serum diluted from 1 in 2.5 to 1 in 1000 with carbolised saline, and incubated at 37° C. for one hour, then put in the ice safe overnight, when the readings were taken, which is the method recommended by Longcope and Rackeman (1918). As no precipitation occurred with any of these six samples of blood and anti-tetanic horse serum when incubated for one hour at 37° C., a further incubation was given on the following day at 52° C. for 24 hours, but no reaction occurred. Five positive results out of the total of six cases of serum sickness were obtained, however, by our method with *S. aureus* antigen as recorded in Table IV.

Table IV.

No. of case	Amount of anti-toxin given	Presence of rash	Strength of reaction (dilution of patient's serum)					
			1 in 5	10	20	40	80	160
1	9,000	Faded	+	+	T*	T	T	0
2	12,000	Onset of rash	++	++	+	+	T	T
3	27,000	Faded	0	T	T	0	0	0
4	39,000	Present	++	+	+	T	T	0
5	45,000	Onset of rash	+++	+++	++	+	+	T
6	15,000	Faded	0	0	0	0	0	0

\* T=Trace.

ON THE PRESENCE OF PRECIPITINS IN NORMAL AND IMMUNE RABBITS' BLOOD WITH FILTERED ANTIGENS MADE FROM CULTURES OF *STAPHYLOCOCCUS AUREUS*.

The first observations on this subject were apparently made by Castellani (1902) who immunised rabbits with cultures of *S. aureus* and found that the immune sera precipitated old cultural filtrates of this organism.

It was an unexpected discovery for us to find that the blood of some normal rabbits in perfect health gave a positive reaction with our *S. aureus* precipitin antigens, and that others failed to do so. Reactions varying in degree from strong to feeble occurred, and further, the strength of the reaction was not a constant feature when repeated observations were made at irregular intervals, although the same antigen and identical technique were employed.

These results are shown very clearly in the following table (V):

Table V. *Normal Rabbits' Blood.*

Rabbit				Dilution of blood serum					
				1 in 5	10	20	40	80	160
1	...	...	...	++	++	+	T*	0	0
2	...	...	...	+	+	T	T	0	0
3	...	...	...	+++	++	+	T	0	0
3	Re-tested 13 days later			+	T	T	T	0	0
4	...	...	...	0	T	0	0	0	0
5	...	...	...	+++	+++	++	+	T	0
5	Re-tested 21 days later			+	+	+	T	T	T
5	"	30	"	T	T	T	0	0	0
5	"	43	"	++	++	+	+	+	T
6	...	...	...	T	T	T	T	0	0
7	...	...	...	T	T	0	0	0	0
8	...	...	...	0	0	0	0	0	0
9	...	...	...	0	0	0	0	0	0

\* T=Trace.

Various points are clearly demonstrated in the above table, namely the intensity of the reaction among normal rabbits, the variation in the degree of the reaction among individual rabbits, and the alteration in the intensity of this reaction when the rabbits are re-tested at intervals of several days. This last observation is clearly demonstrated in the case of rabbit 5.

Having shown that normal rabbits' blood may give a strong reaction, and that this reaction is subject to wide variation, we were fully able to appreciate our results when rabbits were injected with living cultures, filtered cultures of one month's growth, or vaccines of *S. aureus*.

*Staphylococcal Precipitin Reactions*

Table VI.  
*Inoculated Rabbits. Experiment 1.*

Date	Inoculation	Antigen	Results (dilution of serum)					
			1 in 5	10	20	40	80	160
April 29	—	<i>S. aureus</i> (Orpen)	++	++	+	T*	0	0
		<i>S. albus</i>	0	0	0	0	0	—
		Diphtheritic toxin	0	0	0	0	0	—
May 6	50 million <i>S. aureus</i> (Orpen) living							
May 12	—	Filtered <i>S. aureus</i> (Orpen)	++	++	++	++	+	+
May 13	Death from Myelitis							

\* T=Trace.

In the experiment described in Table VI the presence of *S. aureus* precipitin was demonstrated in the blood of the rabbit before it was inoculated, although no reaction occurred with antigens of *S. albus* or with the diphtheritic toxin. A week after obtaining positive results with the uninoculated rabbits' blood, 50 millions of the living emulsion of the same strain of *S. aureus* as furnished in the antigen were injected intravenously. A week later it was found (Table VI) that the strength of the reaction had considerably increased in spite of the fact that the rabbit died on the day following the blood examination from myelitis. Three further experiments are shown to illustrate similar points in the *S. aureus* precipitin reaction (Tables VII, VIII and IX).

Table VII.  
The antigen used in this experiment was *S. aureus* (Orpen)

Date	Inoculation		Results (dilution of serum)						
			1 in 5	10	20	40	80	160	320
July 2	None		T*	T	T	T	0	0	0
" 5	25 million live <i>S. aureus</i> (Orpen) I. V								
" 9	—		++	++	+	T	T	T	0
" 16	50 million		+	+	T	T	T	0	0
" 21	—								
" 23	Death from myelitis								

Table VIII.  
The antigen used in this experiment was *S. aureus* (Nauen)

Date	Inoculation	Results (dilution of serum)					
		1 in 5	10	20	40	80	160
Feb. 27	50 million live <i>S. aureus</i> (Nauen) I. V						
Mar. 1	—	0	0	0	0	0	0
" 5	200 " "	0	0	0	0	0	0
" 8	—	0	0	0	0	0	0
" 16	—	++	++	++	+	+	+
" 21	—	++	++	T	0	0	0
" 24	400 " "						
April 5	—	++	++	++	++	T	0
" 6	Killed						

\* T=Trace.



Table IX.

The antigen used in this experiment was *S. aureus* (Orpen)

Date	Inoculation	Results (dilution of serum)					
		1 in 5	10	20	40	80	160 320
July 2	—	T	T	0	0	0	0
" 5	25 million live <i>S. aureus</i> (Orpen) I. V.						
" 9	—	+	+	T	T	T	0
" 16	50 " "	++	+	+	T	T	0
" 21	—						
" 24	2 c.c. of <i>S. aureus</i> toxin (Orpen)						
" 30	—	++	++	+	+	T	0
Aug. 13	—	+	+	+	+	T	0

In these three experiments the rise in the reaction following the intravenous inoculation of an emulsion of living *S. aureus* using the same culture for the inoculation as employed for the preparation of the filtered antigen is demonstrated.

It is well, however, to realise that fluctuations in the intensity of this reaction occur with the blood of normal healthy rabbits, so that a rise or fall in the reaction may appear apart from inoculation.

*The effect of storing Rabbit Serum on the precipitin reaction.* The following experiments illustrate the loss of potency when immune sera are stored, and how this varied with the method of storage adopted and the specificity of the reaction.

Other experiments with immune sera and various antigens of *S. aureus* were tested and gave somewhat similar results. A serum which gave a very strong precipitin reaction with a *S. aureus* antigen even when diluted to 1 in 1000 with normal saline, was divided into two portions to one of which two drops of chloroform were added and the other was diluted 1 in 5 with formol saline. Each sample of serum was stored in the ice-safe. One month later the chloroformed undiluted serum was the stronger, but the end point

Table X.

1. Immune serum obtained from a rabbit which had been inoculated with emulsions of living *S. aureus* (Nauen) isolated from pus. First tested April 9th, 1923.

Antigen	(Dilution of serum)					
	1 in 5	10	20	40	80	160
<i>S. aureus</i> (Nauen) ...	++	++	++	++	+	0
" (Todman) ...	++	++	++	+	T	0
" (Smith) ...	++	++	++	+	+	0
1 per cent. peptone ...	0	0	0	0	0	0
<i>B. typhosus</i> filtered peptone beef broth	0	0	0	0	—	—
<i>B. coli</i> (Dow) "	0	0	0	0	—	—
" (4869) "	0	0	0	0	—	—

The rabbit serum was then diluted with formol saline (0.1 per cent.), stored in the ice-safe and re-tested on May 15th, 1923.

<i>S. aureus</i> (Nauen) ...	T	T	0	0	0	0
and again re-tested December 12th, 1923						
<i>S. aureus</i> (Nauen) ...	0	0	0	0	0	0

Table XI.

2. Immune serum obtained from a rabbit which had been inoculated with a live emulsion of *S. albus* (osteomyelitis) and tested with filtered antigens prepared from same strain. Serum was collected and divided as follows:

- (1) Pure serum containing 0.1 per cent. formalin. (2) Diluted 1 in 5 with 0.1 per cent. formol saline. (3) Heated at 58° for 20 minutes then diluted with 0.1 per cent. formol saline. All these samples of serum were stored in the ice-safe.

Date		Result Dilution of serum					
		1 in 5	10	20	40	80	160
June 20	Immune serum	++	++	++	++	+	T*
July 4	Stored immune serum						
	No. 1	++	++	+	+	T	T
	" 2	++	++	++	T	T	0
	" 3	++	+	0	0	0	0
July 19	" 1	+	T	T	T	T	0
	" 2	0	0	0	0	0	0
	" 3	0	0	0	0	0	0

\* T=Trace.

had fallen from 1 in 2000 to 1 in 80. These immune sera, however, gradually lose potency in the living animal.

Nuttall (1904) states with reference to haematosera that in the majority of cases these anti-sera deteriorate markedly after three or four months, although some had given good reactions after being sealed in a pure state for several months *in vitro*.

## CONCLUSIONS.

### I. OF METHODS ADOPTED AND OF RESULTS OBTAINED IN STAPHYLOCOCCAL INFECTIONS.

(1) *Staphylococcus aureus* precipitin antigens were prepared with beef broth and 1 per cent. peptone, and were filtered after one month's incubation at 37° C. Filtration was carried out with Berkefeld candles.

(2) Antigens prepared without the addition of peptone to the beef broth were of no value.

(3) All strains of *S. aureus* were found to form efficient antigens, but only one strain of *S. albus* obtained from a case of acute osteomyelitis.

(4) These antigens were satisfactory for a period of at least six months when stored in cupboards at room temperature. The most satisfactory preservative was 0.1 per cent. formalin.

(5) The precipitin reactions were carried out in closed water baths at 37° C. and open water baths at 52° C., but the latter method was preferred, more especially as bacterial contamination was avoided. Readings were taken after 24 and 48 hours.

(6) Heated and unheated sera could be employed, but in most instances the sera were heated at 58° C. for 20 minutes.

(7) Stored sera rapidly deteriorated.

(8) Out of 118 cases of known *S. aureus* infection 43 or 37 per cent. showed a strong or moderate precipitin reaction, 22 or 18 per cent. a weak reaction and 53 or 45 per cent. were negative. The percentage of positive results (55 per cent.) is higher than that found amongst cases where there was no reason to believe that such an infection existed, for amongst this latter group of 98 cases, 42 per cent. were positive. When present, however, this reaction appears to be specific as it was not obtained with filtered antigens made under the same conditions from cultures of *B. coli*, *B. typhosus* and other organisms mentioned except in the presence of co-existing infection.

(9) The blood of many normal rabbits gave a strong or moderate positive reaction with *S. aureus* antigens, but such reactions were found to vary in normal animals and were increased in strength as a result of inoculation of living cultures of *S. aureus*.

## II. OF RESULTS IN SERUM SICKNESS.

Of the 109 cases of serum sickness examined 87·3 per cent. gave a positive reaction with *S. aureus* filtered antigen and of these 79·6 per cent. a well-marked reaction. Viewed as a whole there is no doubt that the precipitin reaction for *S. aureus* in serum sickness is considerably stronger than that obtained in known *S. aureus* infections. The reaction appears also to be specific in the sense that antigens prepared from other organisms in an exactly similar way are of no value. The reaction was not obtained with *S. aureus* grown without peptone and peptone alone showed no antigenic effect. The phenomenon appears to be due to some interaction between the precipitin in the patient's serum and some body which *S. aureus* elaborates from the beef broth and peptone. It was thought that this precipitin in the serum of patients suffering from serum sickness might be bound up or even identical with the precipitins for horse serum which have been described by various workers, but we failed to show this correlation by experiment.

In conclusion we wish to offer our sincere thanks to Dr Foord Caiger for permission to obtain samples of blood from cases of serum sickness at the South Western Fever Hospital.

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# INFECTION WITH COLIFORM BACILLI AS A CAUSE OF RHEUMATOID ARTHRITIS AND CHRONIC RHEU- MATISM: ITS DIAGNOSIS AND ITS TREATMENT BY AUTOGENOUS VACCINES.

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## I. INTRODUCTION.

ALTHOUGH research is still proceeding into the pathological conditions which form the subject of this communication, it is thought advisable to place on record the results obtained up to the present. It was hoped to submit the report of a completed investigation, but each step that has been taken in the experimental production of the diseases concerned, has opened up so many new avenues for research, that this paper must be looked on as only a preliminary report, to be followed by others as material for such accumulates.

It is necessary first of all to attempt to define certain of the terms used in this paper. The designation "coliform bacilli" is applied to organisms isolated from human faeces which belong to the colon-typhoid group of bacilli. In regard to diagnostic terms, those defined by Stockman<sup>1</sup> in relation to rheumatism and arthritis have been mostly followed and are detailed here.

"Acute rheumatism" is a specific fever, the result of a general infection, showing both constitutional and local symptoms, the latter affecting especially the white fibrous tissue of the joints, muscles, heart and other organs.

<sup>1</sup> Stockman. *Rheumatism and Arthritis.*

"Chronic rheumatism" is a condition of chronic inflammation of the white fibrous tissue of fasciae, aponeuroses, sheaths of muscles and nerves, ligaments, tendons, periosteum or subcutaneous tissue, leading to pain, aching, stiffness and other symptoms in the affected part, and is the result of preceding general inflammation, or of local inflammation or injuries.

"Rheumatoid arthritis" is a very chronic disease affecting chiefly the fibrous tissues of the locomotory system, especially involving the joints, causing pain, deformities and crippling, and being non-suppurative and, until recently, unamenable to any specific treatment.

"Osteo-arthritis" is a very chronic disease involving primarily the bone and cartilage, and only secondarily the fibrous tissue of joints, and characterised by marked deformities of the joints resulting from simultaneous bone absorption and bone production.

The first patient treated by the method which will be described in a later section, was sent to the Medical Research Laboratory, Nairobi, by Dr Gilks, now Principal Medical Officer of the Colony, but at that time Resident Surgical Officer at Nairobi Hospital, with a view to determining whether vaccine treatment was possible or advisable for the rheumatoid arthritis from which the patient was suffering. The usual sites of septic absorption were examined and eliminated one by one, until only the intestines remained. It was then that the idea of recovering a number of organisms from the faeces and testing their agglutinability by the patient's serum, suggested itself. Several lactose-fermenting bacilli were isolated on MacConkey's lactose bile-salt neutral-red agar and subcultured on to agar slopes. The purity of these cultures was then tested, and when this was assured, the agglutination reactions of the bacilli with the patient's serum were examined. One organism was agglutinated markedly by a 1 in 100 dilution of the serum, and from it the vaccine was prepared. The initial dose was made very small, under one million bacilli, as it was not known what the reaction might be. Succeeding doses were put up so that each was twice as strong as its immediate predecessor in the series, and were administered at weekly intervals. The first few doses produced no effect, but as the strength of the injections gradually increased, more and more reaction was seen, and was followed by distinct clinical improvement, shown by lessened pain, increased freedom of movement of the affected joints, and the cessation of the progressive character of the disease. Since this patient was seen, more than one hundred cases of disease attributable to infection with coliform bacilli, have been or are being treated, and a significant fact is that most of the new patients have been sent by former sufferers cured or improved by our procedure. The first case treated by our vaccines has now been under observation for five years, and during that time the improvement affected has been maintained.

As time has gone on and brought to the Laboratory a large number of sick persons seeking relief for their various complaints, the conditions in which agglutinable coliform bacilli may be isolated from the faeces have been

found not to be confined to rheumatoid arthritis as was believed at first, but to include cases of chronic rheumatism and other allied diseases, almost all being apparently amenable to vaccine treatment.

In the present paper it is our intention first to describe briefly the technique employed in the examination of patients for infection by coliform organisms and in the preparation of the vaccine, then to give an account of the clinical effects of the treatment, next to discuss shortly experimental facts discovered in the laboratory, and finally to attempt to formulate a working hypothesis to explain our results on the basis of our present knowledge.

## II. TECHNIQUE EMPLOYED IN DIAGNOSIS AND IN THE PREPARATION OF AUTOGENOUS VACCINES OF COLIFORM BACILLI.

When a patient complains of symptoms, or on examination shows signs suggesting the presence of an infection with an organism of the coli group, a sample of his blood is withdrawn from a vein to provide serum for the agglutination test and he is instructed to send to the Laboratory as early as possible a sample of his faeces. When the latter arrives, a small loopful of it is inoculated into each of two tubes containing bile-salt peptone water (sodium taurocholate 0.5 per cent.: peptone 1.0 per cent.). These tubes are incubated at 37° C. for 24 hours, and then a loopful from each is spread over the surface of neutral-red bile-salt lactose peptone agar in Petri dishes in such a manner as to secure a large proportion of isolated colonies, the number of plates required for each loopful to obtain this result depending on the technical skill of the operator. These plates are incubated at 37° C. for 24 hours, and then two or three representatives of each variety of colony seen are picked off and inoculated into tubes of nutrient broth, which after incubation for 24 hours at 37° C. form the cultures to be tested for agglutination with the patient's serum. Four methods of conducting this test have been employed at various times. The original one used was that described by Broughton-Alcock<sup>1</sup>. This did not prove very satisfactory in practice, probably owing to the delay experienced in the reaction with coliform bacilli. The microscopic hanging-drop method using a platinum loop to make the dilutions, was then adopted, but was found to give rise to anomalous results which can reasonably be attributed to the inaccuracy of the method of dilution used in this test, a surmise supported by the discovery that when dilution of the serum, and the mixing of the diluted serum with the broth culture are carried out in bulk by means of a calibrated dropping pipette, and the hanging-drop taken from the resulting mixture, the findings conform very closely to those obtained with Neisser's or Dreyer's technique. The methods at present in use are those described by Neisser<sup>2</sup> and by Dreyer<sup>3</sup>. It is probable that Neisser's method is theoretically open to objection, but as in a long series of tests, the results

<sup>1</sup> Broughton-Alcock. *Journ. Royal Army Med. Corps*, xxx. 424.

<sup>2</sup> *Med. Res. Council* (1920). Special Report Ser. No. 51. p. 111.

<sup>3</sup> *Ibid.* No. 51, p. 128.

obtained with it have been comparable with those given by Dreyer's method at serum dilutions considered significant of infection, from the practical point of view it does not seem to be a matter of great importance which method is used. Of course when the end-titre of a serum for a particular bacillus is in question, it is essential to employ Dreyer's technique, and here it must be emphasised that when this method is used, the bacillary emulsions must be grown in veal broth, and must be very thin, that the flocculation obtained is much finer than with typhoid cultures, and that the agglutination tubes must be incubated for 5 hours at 50–55°C. As in all our cases a titre of 1 in 90 has been found to be constantly present in the patient's blood for the coliform organisms considered responsible for the infection, this dilution of serum has been adopted as the lowest significant in the test, the usual controls of course being set up.

The cultures in broth of those organisms which show agglutination at the significant titre with the patient's serum, are again plated out on neutral-red bile-salt lactose peptone agar to test for purity. If the cultures are pure, an isolated colony from each is picked off and inoculated on to an agar slope, but if the cultures are mixed, each variety of colony that appears, must be re-tested for agglutination with the patient's serum, and those which show agglutination, re-examined for purity. When pure cultures of the agglutinating bacilli are obtained, a 24 hours old growth of each variety on an agar slope is emulsified in a small quantity of a freshly prepared 0.5 per cent. solution of carbolic acid in normal saline. The emulsions so obtained are mixed in amounts directly proportional to the agglutinability of each variety of bacillus in a sterile bottle containing sterile glass beads, and then shaken in a mechanical shaker for an hour or more until the bacteria are uniformly distributed throughout the liquid. The emulsion is allowed to stand for 48 hours, after which a loopful is subcultured on to an agar slope to test it for sterility. As soon as the vaccine is sterile, it is standardised by the method described by Brown and Kirwan<sup>1</sup> to contain 0.1 milligramme of dried bacilli per cubic centimetre, and placed in a small sterile bottle which is then closed by a sterile rubber cap, and sealed by a gelatin solution. The method of dosage recommended at present is an initial injection subcutaneously of 0.1 c.c. (equivalent to 0.01 mgm. of dried bacilli), subsequent doses being usually increased by 25 per cent. until a maximum of 2.0 c.c. (equivalent to 0.20 mgm. dried bacilli) is reached. The vaccine is administered at intervals varying with the amount of reaction shown to each dose by the patient, a period of four clear days after all reaction from the previous injection has disappeared, being ordinarily sufficient.

<sup>1</sup> Brown and Kirwan (1914–15). *Indian Journ. Med. Res.* II. 763.



### III. THE CLINICAL RESULTS OF TREATMENT BY AUTOGENOUS VACCINES OF COLIFORM BACILLI.

In Table I will be found a list of all cases treated by our vaccines of which information as to the original clinical condition and the final result is available. At the foot of this table will be found a summary of the results, not only of the cases detailed, but also of all other cases treated by us, these latter being found grouped under three headings, "Result unknown," "Cases insufficiently treated," and "Cases still under treatment."

Table I.

Case No.	Diagnosis	Result	Case No.	Diagnosis	Result
1	Rheumatoid Arthritis	Greatly improved	48	Rheumatoid Arthritis	Greatly improved
2	Rheumatoid Arthritis	Improved	49	Chronic Rheumatism and Neuritis	Cured
3	Rheumatoid Arthritis	Greatly improved	50	Chronic Rheumatism and Sciatica	Improved
4	Rheumatoid Arthritis	Cured	51	Chronic Rheumatism and Sciatica	Cured
5	Rheumatoid Arthritis	Greatly improved	52	Bacilluria, ( <i>B. coli</i> )	Cured
6	Rheumatoid Arthritis	Greatly improved	53	Rheumatoid Arthritis	Greatly improved
8	Chronic Rheumatism	Cured	54	Chronic Rheumatism	Cured
9	Chronic Rheumatism	Cured	55	Sciatica	Greatly improved
11	Rheumatoid Arthritis	Cured	56	Rheumatoid Arthritis	Cured
12	Rheumatoid Arthritis	Greatly improved	57	Chronic Rheumatism and Neuralgia	Cured
14	Chronic Rheumatism	Cured	58	Bacilluria, ( <i>B. coli</i> )	Cured
15	Acute Rheumatism	Slightly improved	60	Rheumatoid Arthritis	Greatly improved
16	Chronic Rheumatism	Cured	61	Pyrexia	Improved
17	Osteo-arthritis	No improvement	62	Colitis and Chronic Rheumatism	Cured
20	Osteo-arthritis	Slightly improved	63	Acute Rheumatism	Cured
21	Rheumatoid Arthritis	Cured	65	Chronic Rheumatism	Greatly improved
24	Chronic Rheumatism	Cured	66	Chronic Rheumatism and Bacil- luria, ( <i>B. coli</i> )	Cured
25	(?) Gonorrhoeal Rheumatism	Improved	67	Chronic Rheumatism	No improvement
27	Rheumatoid Arthritis	Greatly improved	68	Colitis	Cured
28	Chronic Rheumatism	Cured	69	Chronic Rheumatism and Bacil- luria, ( <i>B. coli</i> )	Greatly improved
29	Chronic Rheumatism and Sciatica	Cured	70	Rheumatoid Arthritis	Cured
31	Osteo-arthritis	Not improved	71	Rheumatoid Arthritis	Greatly improved
32	Infected gunshot wound of leg	Cured	72	(?) Gonorrhoeal Rheumatism	Improved
34	Chronic Rheumatism	Cured	73	Chronic Rheumatism and Bacil- luria, ( <i>B. coli</i> )	Greatly improved
36	Rheumatoid Arthritis and Sciatica	Greatly improved	74	Chronic Rheumatism	Cured
37	Arthritis	Not improved	75	Lumbago and Sciatica	Greatly improved
38	Rheumatoid Arthritis	Cured	77	Lumbago	Cured
39	Chronic Dyspepsia	Cured	78	Rheumatoid Arthritis	Not improved
40	Chronic Rheumatism	Improved	79	Chronic Rheumatism	Cured
41	Rheumatoid Arthritis	Greatly improved	80	Chronic Rheumatism	Cured
42	Chronic Rheumatism	Greatly improved	83	Lumbago	Cured
44	Membranous Colitis	Cured	85	Rheumatoid Arthritis	Cured
45	Chronic Rheumatism	Cured	86	Colitis	Not improved
46	Bacilluria, ( <i>B. coli</i> )	Cured	87	Colitis and Cystitis	Greatly improved
47	Bacilluria, ( <i>B. coli</i> )	Cured	88	Chronic Rheumatism	Greatly improved

#### SUMMARY. (Results of all cases treated up to June, 1924.)

Disease	Total cases treated	Results of treatment						Insuffi- ciently treated	Under treat- ment
		Cured	Greatly im- proved	Im- proved	Slightly im- proved	Not im- proved	Result un- known		
Abscess	1	—	—	—	—	—	—	—	1
Arthritis	1	—	—	—	—	1	—	—	—
Bacilluria, ( <i>B. coli</i> )	7	4	—	—	—	—	—	—	3
Colitis	4	2	1	—	—	1	—	—	—
Constipation, Chronic	1	—	—	—	—	—	—	1	—
Dyspepsia, Chronic	1	1	—	—	—	—	—	—	—
Infected gunshot wound of leg	1	1	—	—	—	—	—	—	—
Osteo-arthritis	4	—	—	—	1	2	—	1	—
Pyrexia	1	—	—	1	—	—	—	—	—
Rheumatism, Acute	4	1	—	—	1	—	—	—	2
Rheumatism, Chronic	47	20	7	2	—	1	1	6	10
Rheumatism, (?) Gonorrhoeal	2	—	—	2	—	—	—	—	—
Rheumatoid Arthritis	22	7	12	1	—	1	—	1	—
Diagnosis unknown	15	—	—	—	—	—	9	2	4
Total	111	36	20	6	2	6	10	11	20

A short explanation must here be given as to what is meant by certain of the headings used in the table.

"Cured" means a complete return to health of the previously affected parts as far as can be determined by clinical examination.

"Greatly improved" means that the patient's condition has become as normal clinically as was permitted by the permanent pathological changes present when treatment was commenced.

"Improved" means that there has been complete cessation of the progressive character of the disease, and marked amelioration of the physical signs and symptoms.

"Slightly improved" means that there has been some definite though not great improvement in the patient's condition.

"Not improved" means that the vaccine has produced no apparent change in the progressive character of the disease.

"Result unknown" means that the practitioner in charge of the case has failed to supply information as to the results of the vaccine.

"Cases insufficiently treated" are those in which the patient through carelessness or through dissatisfaction at the failure of the vaccine to cure in a few doses, has only received two or three injections at irregular intervals. Certain patients who failed to come for their vaccines when these were ready, are included in this class.

The diagnoses used in the table conform to those set out in the introduction to this paper.

It is proposed in this section to give a detailed description of a few cases treated by autogenous coliform vaccines which either demonstrate most clearly the results commonly obtained, or are important as illustrating points of significance in the elucidation of the pathogenic properties of coliform bacilli.

The following three cases have been selected as typical of the results of autogenous coliform vaccines in rheumatoid arthritis.

*Case 1.* Male. Adult. Came under observation in August 1919. His history was that rheumatism began in 1913 in the shoulders, wrists and ankles. He was treated with salicylates, potassium iodide, liniment and radiant heat. Slight local improvement resulted, but the general condition remained unchanged. Endocarditis developed at the end of 1913, and the patient was in bed for three months, and off duty for six months. He was advised to come to East Africa in 1917, at which time he had considerable pain in, but little fixation of, his joints. After his arrival in Kenya Colony, the pain became worse, and the joints, especially those of the left shoulder and the right wrist, became more or less fixed. In 1918 the patient came under the care of Dr Gilks for the first time. The left shoulder-joint was then markedly limited in movement, and much creaking was observable on attempting to move it. The shoulder muscles were very wasted. The right wrist joint was swollen, the swelling involving the carpal joints as well, while there was considerable bony deformity and a great thickening of the periarticular fibrous tissue. Movement was practically absent. The interphalangeal joints of the fingers showed spindle-shaped swelling with much restriction of movement, though bony deformity was absent. Other joints were affected less markedly, though in most there was considerable pain, especially in the ankles. In August 1919 this patient was seen at the Laboratory. There was no evidence of pyorrhoea, nor was there a sign of any other source of septic absorption, except that suggested by the chronic constipation complained of by the patient. His faeces were examined, and from them *Bacillus coli communis* and *B. paragrünthali* were isolated and found to be agglutinated by the serum of the patient in a dilution of 1 in 100. A vaccine was prepared from these two organisms. The patient improved greatly as the result of treatment with

this vaccine. His general health became better, he put on fat, his appetite returned, the mental depression disappeared, and the muscles recovered much of their tone. The movements of the left shoulder-joint increased so that the left hand could touch the right ear over the top of the head. The movements of the right wrist joint did not improve except slightly, but the finger joints became normal in shape and movement. The disability which remained was very largely due to permanent pathological changes in the joints present when treatment was commenced and which vaccine therapy could not be expected to remove. At the present time the patient is able to play tennis and ride a motor-cycle. Slight recurrences of pain have occurred on two occasions subsequent to the first vaccine treatment (in December 1921 and December 1922). Both were successfully treated by vaccines prepared from fresh agglutinable coliform bacilli isolated from the patient's faeces. There has been no relapse in the clinical improvement shown in the affected joints although it is now five years since the first vaccine was given.

*Case 5. Female. Adult.* Came under observation in May 1920. The patient was 53 years of age. She had had rheumatic fever 12 years previously, and had been subject to twinges of rheumatism all her life. In June 1915 she developed pain, stiffness and swelling in the right knee, and had to walk with a stick. She had marked pyorrhoea at the time. She returned to England, and was treated at Buxton. All her teeth were removed, and the rheumatism improved. On the return voyage, the weather was very bad, and the left knee became affected. The disease spread to her right hand, then to both ankles and later to other joints. The patient gradually became more crippled, and in May 1920 she could only shuffle about with a stick. Her back was bowed, and the stoop on walking was very marked. Both knees were very swollen owing chiefly to thickening of the periarticular tissues and of the synovial membrane, the fluid present in them not being great in quantity. The right ankle was swollen from the same causes. Both hands were swollen and showed ulnar flexion, more marked in the right one, while the fingers of this hand were flexed and ankylosed. Anti-rheumatic treatment with salicylates, iodides and radiant heat had no effect. From this patient's faeces in May 1920 a coliform bacillus was isolated which was agglutinated by the patient's serum in high dilution. A vaccine was therefore prepared from this organism, and steady improvement followed treatment with it. Pain ceased almost immediately, and at the conclusion of the course the patient could walk two miles with ease, and the stick was discarded. She now could carry out her full school duties. The back became straight, and there was no stoop on walking. Some slight swelling of the knee persisted, and flexion of that joint only improved to three-quarters of normal, though extension was full. The deformity of the right hand remained, but the use of it was almost completely regained, and the ulnar flexion became less pronounced. The left hand still showed enlargement of the metacarpo-phalangeal joints of the index and middle fingers. The patient's general health greatly improved, although the housing and other environmental conditions of her life remained unchanged. Since treatment ceased four years ago, the improvement has been maintained, indeed some of the deformities have become less marked, while there has been no recurrence of pain or disability.

*Case 70. Female. Adult.* Came under observation in July 1923. There was a family history of lumbago, chiefly amongst the patient's brothers and sisters. Since she was 18 years old, she had had acute attacks of lumbago at intervals of about three years, while between attacks she had had stiffness and limitation of movement of the joints of the back. In 1918, she had neuritis in the right arm causing much pain, and great wasting of the muscles. A short time prior to coming to the Laboratory, the patient noticed that the interphalangeal joints of the right hand had become thickened and painful, and were subject to exacerbations during which the affected joints became reddened and swollen. The patient was unable without considerable difficulty to open her hands after gripping an object. She complained of chronic constipation, and of loss of appetite and sleeplessness. On examination the affected joints were found to be enlarged due to thickening of the

periarticular tissues, and on passive movement they gave slight crepitation, while the patient complained of creaking sensations. Slight ulnar flexion was observable in the right hand. *B. pseudocoloides* A. was isolated from the patient's faeces, and was found to be agglutinated by the patient's serum in a dilution of 1 in 300. A vaccine was prepared from it, and after the third dose the patient's appetite and sleeplessness improved. After ten injections she could do everything with her right hand without pain or disability, except that after exerting much force, the third or even all the fingers sometimes became locked, and had to be forced open. The local condition in the hand was steadily improving while the patient's general health was very much better. In March 1924 she came to report that she had never felt so well in her life, and that the right hand had completely recovered, being free from any pain or disability and having no tendency to locking of the fingers after closing the hand forcibly. The periarticular thickening had entirely disappeared, and the hand looked normal. The patient remarked that the improvement due to the vaccine continued for some considerable time after the injections ceased.

The following two cases are reported as examples of patients who have had several relapses. Fortunately such cases are uncommon.

*Case 12.* Female. Adult. Came under observation in November 1920. The patient had a history of increasing pain for three years in the hands and feet, which was so great that, coupled with the disability attendant on stiffness of the joints of the hands, it rendered her work on her farm impossible of execution. On examination the hands were almost completely crippled, and could not be closed. The interphalangeal joints of the hands showed spindle-shaped swellings due to thickening of the periarticular tissues, while the metacarpo-phalangeal joints were enlarged from the same cause. There was marked limitation of movement in all the affected joints, and ulnar flexion of both hands. A coliform bacillus was isolated from the patient's faeces, which was agglutinated by her serum in high dilution, and from it a vaccine was prepared. After treatment with this vaccine, the patient remained free from pain for ten months. The periarticular thickening of the affected joints diminished, so that the deformity occasioned by this, and the disability due to limitation of movement almost completely disappeared. In December 1921 there was a slight recurrence of pain and stiffness in the fingers. The faeces were again examined, and a coliform bacillus Type IV (see Table II) isolated which was agglutinated by the patient's serum in a dilution of 1 in 100. A vaccine prepared from this bacillus led to disappearance of pain and stiffness for eight months. In September 1922 there was another slight recurrence of the same symptoms, but these disappeared completely after a vaccine prepared from *B. pseudocoloides* A, isolated from the patient's faeces and agglutinated by her blood serum in a dilution of 1 in 200, had been administered. In July 1923 the patient asked for another vaccine to be made to take with her to England for fear a relapse should occur during her absence from Kenya Colony. A coliform bacillus Type IV was again isolated from her faeces, and found to be agglutinated by her blood serum in a dilution of 1 in 100. The vaccine was made but no occasion arose for its use. While in England the diagnosis of the disease as rheumatoid arthritis was confirmed, and as X-ray examination of her teeth showed these to be normal, she was persuaded to have her enlarged tonsils removed. The patient stated on her return to Kenya in May 1924 that her condition had been steadily improving since the last vaccine administration, and that the tonsillectomy had made no appreciable difference. At her request her faeces were once more examined, and a *Bacillus pseudocoli* isolated therefrom which was agglutinated by a 1 in 250 dilution of the patient's serum. A vaccine was made up from this organism, but no further information regarding this patient has since come to hand.

*Case 27.* Female. Adult. Came under observation in August 1921. The patient had a history of attacks of rheumatism for the past 12 years affecting almost all the joints of the body. When these attacks occurred, the patient became a complete cripple, and was

unable to move any of the joints affected. The rheumatism was markedly worse when the rainy season began, and more especially so, when the patient was nursing a baby. The small joints of the hands had only been affected for the preceding three months. Clinically during an attack there was swelling of the affected joint, and fixation of the latter by involuntary muscular contraction, while even the slightest passive movement caused intense pain. Ordinarily the attack would confine itself to one joint for a few days, then pass on to another, leaving the original joint with the periarticular tissues more or less thickened. Rarely was there an interval when no joint was affected. There was a long history of chronic constipation. On examination of the patient during a quiescent interval, there was found to be a thickening of the periarticular tissues of the ankle joints, which were swollen and painful on movement. There was a considerable dropping of the arch of the foot on both sides, the result seemingly of these rheumatic attacks. The wrists showed the same periarticular tissue thickening as the ankles, though not to so great a degree. The interphalangeal articulations of the index and middle fingers of the right hand, and of the index, middle and third fingers of the left hand showed spindle-shaped swellings, due mainly to thickening of the periarticular fibrous tissue. The other joints of the body showed no gross change detectable by clinical examination. From the patient's faeces was isolated a coliform bacillus Type XVIII which was strongly agglutinated by the patient's serum when diluted 1 in 100. A vaccine was made from this bacillus, and administered to the patient. The result was most satisfactory, as all symptoms disappeared for seven months, and all the clinical signs of the former pathological condition practically cleared up. At the end of March 1922, however, there was a slight recurrence of pain in one arm, an event coinciding with the onset of the long rainy season. *B. pseudocoli* was then isolated from the patient's faeces, and this was agglutinated by the blood serum in a dilution of over 1 in 300. A vaccine was prepared from this organism, and its administration resulted in complete disappearance of the symptoms which did not recur till June 1923 in spite of the fact that the patient while on vacation in England, experienced a very cold and inclement winter. In January 1923 the patient became pregnant, and in June of that year following some damp and cold weather at the end of the rainy season, she suffered some slight twinges of pain in the arm. The patient's faeces were examined again, and three bacilli, *B. pseudocoli*, *B. metacoloïdes* and a coliform bacillus Type VII, were isolated, and were agglutinated by a dilution of 1 in 100 of the patient's serum. A vaccine was made up from these three organisms, but not then administered in view of the pregnancy and the disappearance of the rheumatic symptoms. A month after the baby was born, the patient who was suckling the infant, began to suffer severely again from rheumatism, and then remembered that after each of her two previous confinements, she had had bad attacks of this disease while feeding the babies. The vaccine prepared in June of this year was now given with good effect, but although the pain almost completely disappeared, the swelling of the interphalangeal articulations remained, and was accompanied by considerable stiffness. For six weeks after the completion of this vaccine course, the patient, who was still breast-feeding the baby, was much better, but after this period, her rheumatic symptoms gradually got worse, more especially so in the feet. A further examination of the faeces was advised in June 1924 and *B. pseudocoli*, and an unidentified coliform bacillus were isolated, and found to be agglutinated by the patient's serum in a dilution of 1 in 100. A vaccine was prepared from these bacilli and administered to the patient, and a report received in October 1924 states that she has completely recovered, and now shows no clinical symptoms or signs whatever.

This case is of interest as showing both the persistence of a coliform organism, and its association with a variety of other allied coliform bacilli on different occasions as pathogenic agents, and also from the correlation of the rheumatic attacks and lactation. It seems probable that the rheumatic symptoms complained of in June 1923 are explicable by the coincidence of pregnancy with inclement climatic conditions. The existence of each sepa-

rately, apparently induced no attack of rheumatism, as witness the absence of the disease in England, and again after June 1923 when the weather had improved, though the pregnancy continued. The strain of lactation however broke down the patient's resistance, and permitted a re-infection with one of the original organisms in association with a new coliform bacillus.

The four cases reported below are described because they illustrate points of importance in the elucidation of the pathology of the group of diseases dealt with in this paper.

*Case 32. Male. Adult.* Came under observation in October 1921. During the late war he received some shrapnel wounds in the back, and a severe gunshot wound of the right thigh causing comminution of the middle third of the femur. The patient was in hospital in England for two years, but left finally with a sound femur albeit a stiff knee, the surgeons fearing to attempt the breaking down of adhesions in the joint lest the healed sinuses from the gunshot wound should become inflamed again. Amongst other treatment during his stay in hospital, he received a stock coli vaccine, as the pus from the wound was largely the result of an infection with coliform organisms. This vaccine apparently produced little effect. The patient came to Kenya Colony at the end of 1919, but a few days after arrival had the misfortune to catch his foot in an open drain, and in falling heavily on the ground, to break the previously injured femur close to the site of the original comminution. Although the fracture was not compound, the tissues around the old wound became inflamed, and some of the former sinuses began once more to discharge pus, which occasionally contained tiny sequestra of bone. The inflammation had periods of subsidence or quiescence, and periods of recrudescence, this state of affairs persisting for about 12 months. During this time several fragments of bone were removed at operation, and at the end of it, the physical signs pointed to an abscess situated close to the bone. Strange to say, the fracture, in spite of the inflammation, had reunited. The abscess was opened by an exploratory incision, and the pus, which contained no bony sequestrum, evacuated. This pus on bacteriological examination only showed *Staphylococcus albus*. Unfortunately these surgical measures did not give the measure of relief expected, nor did a staphylococcal vaccine, and the patient's condition rapidly became worse as shown by the appearance of a slight quantity of albumin in the urine, coupled with a decided loss of weight and with progressive weakness. It was then suspected that the patient might be still suffering from the original coliform infection acquired when he was wounded, although the pus from the discharging sinuses only showed an infection with *Staphylococcus albus*. Recourse had therefore to be had to the measures adopted by us for the preparation of vaccines for coliform infections. The patient's faeces and urine were examined; the latter was sterile, but the former yielded *B. pseudocoli* which was agglutinated by the patient's serum in a dilution of 1 in 250. From this organism a vaccine was prepared, the administration of which caused immediate improvement in the patient's condition. The appetite returned, and weight increased; the sinuses healed up, and the albuminuria disappeared. This improvement has been maintained without a sign of relapse up to the present, and the patient plays tennis regularly, and golf occasionally.

This case suggests that the original coli infection had never actually died out, while from the high agglutination titre it might be concluded that the coliform bacillus isolated from the faeces was identical with that causing the original infection. If this was so, the failure of the stock coli vaccine is understandable, and the case supports the view that if a patient has in his blood a certain concentration of agglutinins for a coliform bacillus isolated from his faeces, he is suffering from an infection with, or from the absorption of toxins elaborated by that coliform bacillus.

Case 37. Male. Adult. Came under observation in December 1921. He was a man aged about 40, who had led a very active life up to a few years previously. He had had gonorrhoea when a youth, but had never suffered from a relapse. There was no history of syphilis, either congenital or acquired. The illness for which he sought relief, began in 1919 with pain in the knees. It was thought by his medical adviser to be due to the original attack of gonorrhoea, and it was suspected that the prostate, which was enlarged and hard, was the seat of a latent infection with gonococci. Prostatic massage was therefore instituted, but was followed immediately by synovitis of both knee-joints, at first only slightly painful and remaining but a few days, then disappearing to reappear a week later. A definite periodicity in the incidence and subsidence of the synovitis of the knees became established, which was unaffected by any treatment. The patient then returned to England, where he was treated by many doctors and by many different methods, including the administration of gonococcal vaccine, with very little, if any, improvement in the disease. Business necessitated his return to Kenya Colony. On arrival there it was noted that the knees were swollen for three days, and more or less normal for three days, that there was a definite rise of temperature associated with the synovitis, and that there was also a coincident synovitis of both ankle-joints, and pain in the right shoulder. At the time of the exacerbation of pain and swelling of the knees, there was a definite tendency to increased frequency of micturition. These were the symptoms when the patient first came under our observation. The physical signs present in an attack were extreme distension of the knee-joints, the skin over which seemed to be on the point of bursting, accompanied by tenderness so great that the patient was confined to bed, slight synovitis and tenderness of both ankle-joints, and slight creaking in the right shoulder-joint on passive movement. The temperature rose to 100–101° F. The urine passed during an attack was thick and cloudy. During the quiescent period no pathological changes could be made out by clinical examination in the ankles or shoulder, but there was a decided thickening of the synovial membrane of the knee-joints, though very little of the periarticular fibrous tissues, and none of the bony structures of the joints. There was a certain amount of lateral movement in both knee-joints due undoubtedly to the overstretching of the lateral ligaments of those articulations. The prostate was still enlarged and hard. The patient's general condition was fair, though lack of exercise had rendered him fat and flabby, and the constant attacks of pain had induced a condition of neurasthenia. The Wassermann reaction was negative on three occasions. The temperature curve, the periodicity of the attacks, and the increased frequency of micturition suggested a bacterial infection, but no obvious focus for this could be found, as there was no pyorrhoea, and both the synovial fluid from the knees and the secretion from the prostate were sterile. Gonococcal vaccines had been tried recently with no effect whatever. The faeces and urine of the patient were therefore examined by the technique described in the preceding section, and three bacilli, *B. pseudocoloides* A, *B. pseudocoli* and *B. coli communis* were obtained from the faeces, and *B. metacolooides* from the urine, all of which were agglutinated by a 1 in 150 dilution of the patient's blood serum. A vaccine was prepared from these bacilli, and its use was followed by complete disappearance of the frequency of micturition, a diminution in the pain in the knees, and a slight delay in the onset of the synovitis of the left knee. Unfortunately this last result had the effect of rendering the presence of synovitis in one or other knee practically constant, and can be traced in all probability to the too frequent administration of the vaccine, and the production of too violent reactions in a patient who was apparently hypersensitive. Later on it became obvious that the initial improvement produced by the vaccine had not been maintained, and a further investigation of the patient's secretions and excretions was advised in May 1922. The prostatic fluid was again sterile, but the faeces yielded *B. pseudocoli*, and three coliform bacilli Types II, V and VII, and the urine on one occasion *B. pseudocoli* and a coliform bacillus Type XXI, and on another occasion, a month later, during an attack of increased frequency of micturition, *B. coli communis*. All these bacilli

were agglutinated by the patient's serum in a dilution of 1 in 200. A vaccine was prepared from these organisms, and the patient who meanwhile had decided to return to England, took it with him. No further report on this patient has yet been received.

This case is of great interest in spite of the failure of the vaccine. The symptoms all pointed to a bacterial infection of some kind. Our investigations indicated that the intestinal tract was the most probable source of the bacteria, this view receiving support from the finding of the same agglutinable coliform bacilli in the urine as in the faeces. Possibly had the gonococcal vaccine been combined with the coliform one, more favourable results would have been seen. A very important fact to be noted in this case is the finding of atypical coliform bacilli in the urine, and also the variation of type of the agglutinable coliform bacilli isolated from the faeces even within a comparatively short period. An explanation for this latter fact might be that variation in cultural reactions in the case of the coliform bacilli does not necessarily imply a fundamental difference in species, or else that a bacillus against which the body has acquired immunity, can change its antigenic properties, this being accompanied by a change in cultural reactions in certain cases, or else that when the body has become protected against one bacillus, a very closely allied species of bacteria may acquire pathogenic properties and carry on the disease.

*Case 40.* Male. Adult. Came under observation in February 1922. The patient had suffered for some years with a fistula-in-ano, but some time prior to its development, there had been pain and stiffness of the vertebral column, more marked in the cervical and thoracic regions, stiffness but no pain in the small joints of the fingers of both hands, and discomfort in the head almost amounting to headache. Patient had suffered from several attacks of malaria since he had been in Africa. At the time the patient first came under observation, these complaints were confined to the stiffness in the fingers, the stiffness and pain in the neck, and a very considerable degree of malaise. Soon after this, the fistula-in-ano gave rise to an ischio-rectal abscess, and coincidently there was a distinct improvement in the rheumatic symptoms in the hands. Later however the pain and stiffness reappeared, and the patient requested vaccine treatment. At this time the fistula-in-ano had not completely healed. The patient's faeces were examined, and *B. coli communis* isolated, while at the same time the secretion of the fistula yielded a coliform bacillus Type V, both these organisms being agglutinated by the patient's blood serum in a dilution of 1 in 600, other bacilli from the same sources not being agglutinated even in a dilution of 1 in 50. A vaccine was prepared from these two agglutinable bacilli, and its administration led to very considerable improvement, though not to a complete cure. A further examination of the faeces was therefore made, and a coliform bacillus Type VII, *B. coli communis*, and *B. paragrünthali* were isolated, and were agglutinated by the patient's serum in dilutions of 1 in 200, 1 in 400 and 1 in 800 respectively. A vaccine was prepared from these three bacilli, and its employment was followed by healing of the fistula-in-ano, and the complete disappearance of the rheumatic symptoms for some months. In September 1923 there was a slight recurrence of pain and stiffness in the cervical and thoracic regions of the vertebral column. The faeces on examination yielded *B. coli communis* and a *B. paragrünthali*, both of which were agglutinated strongly by a 1 in 100 dilution of the patient's serum, higher dilutions not being tested. A third vaccine was prepared from these bacilli, but apparently from the report received from the patient, its use had little or no effect on the symptoms. The hands recovered completely after the first batch of vaccine was given.



The points which this case emphasise are, firstly that in some instances the patient's blood serum agglutinates these coliform bacilli in very high dilutions, and secondly that where a vaccine does not cause a complete cure, it is often due to the failure to isolate all the coliform bacilli responsible for the infection, though here it must not be forgotten that, more especially in cases where the vaccine first of all causes marked improvement and later fails to maintain it, it is always possible that a new infection with a closely allied species of coliform bacillus may have supplanted the bacillus against whose action the body is now protected, or that by changing its antigenic properties, the originally infecting bacillus may have managed to evade the new defences set up in the body. A possible portal of entry for the coliform bacilli causing the original symptoms was the fistula-in-ano, which may have been in existence some considerable time before it caused symptoms. It is interesting to note in this patient the disappearance of the rheumatic symptoms when the ischio-rectal abscess appeared, a phenomenon which may perhaps be attributed to autoinoculation by the bacteria or bacterial toxins in the contents of the abscess.

*Case 46.* Male. Adult. Came under observation in May 1922. This patient was first seen in 1917 when he complained of pain in the lumbar region, loss of weight, and "thick" and very offensive urine. There was then slight albuminuria, but the urine contained no casts. His symptoms were relieved by the exhibition of alkalies. Shortly afterwards the patient left for England, and on arrival there his former symptoms recurred, and as he was undoubtedly suffering from an infection of the urine by a coliform organism, he was given a stock coli vaccine which gave great relief; indeed there was no recurrence of the disease till the spring of 1922. In May 1922 he was complaining of pain in the right loin over the kidney, dysuria and greatly increased frequency of micturition. Drugs this time brought no relief. On examination the right kidney was enlarged and tender, and the urine, when passed, was very cloudy. From the urine *B. coli communis* was isolated, and was agglutinated by the patient's blood serum in a dilution of over 1 in 100. A vaccine was prepared from this bacillus, and its use resulted in a complete cure. There was no recurrence of the disease till the spring of 1924, when all the previous symptoms began to return, and these had become as bad as ever by the time the patient sought relief in June 1924. On this occasion it was discovered that the patient's wife had been suffering from a coli infection of the urine for 12 years, that is to say her infection existed before that of her husband. An investigation of the organisms isolated from the urines of both patients was undertaken, but no report as to the results obtained had been received up to the time of writing.

This case is of interest in that both husband and wife were sufferers from an infection with coliform bacilli. As another instance of this family incidence, it may be noted that Case 73 is the mother of Case 58.

#### IV. AGGLUTINATION REACTIONS FOR AUTOGENOUS COLIFORM BACILLI SHOWN BY "NORMAL" PERSONS.

Eight patients suffering from arthritis, when examined by the technique described in Section II, did not show in their faeces coliform bacilli which were agglutinable by their sera. Further examination and enquiry revealed that in four instances the disease was gonorrhoeal in origin, and in a fifth case

was due to tuberculosis. Considering the number of cases seen, however, the percentage of positive agglutination results has been so high as to raise a doubt in our minds as to whether the agglutination on which our technique was based, was specific or not. To determine this question it was decided to examine a series of "normal" cases. When the task was begun, it was found to be extremely difficult to obtain "normal" cases, as nearly every person examined claimed to have or to have had "rheumatism" in some form or another. Fifty-four patients who were in hospital for treatment of non-intestinal diseases, were found clinically free from any evidence of an infection with a coliform bacillus, and the faeces of these persons were investigated in the manner described in the second section of this paper. In only two instances were coliform bacilli found which agglutinated with a 1 in 90 dilution of the patient's serum, and in one of these the agglutination was so poor that had the faeces been under examination with a view to vaccine treatment, the result would have been discarded as unreliable. Unfortunately there was no opportunity of examining these two patients again as they left hospital before the tests were completed.

Dudgeon, Wordley and Bawtree<sup>1</sup> have stated that out of 66 "normal" individuals examined for agglutinins for a typical *B. coli*, only five showed any in a serum dilution of 1 in 50, using Dreyer's method. In a later paper<sup>2</sup> the same authors report that in a further 104 "normal" persons believed free from a coli infection, sera from 22 cases only gave a positive agglutination reaction at a dilution of 1 in 50, and two of these on further investigation showed evidence of a probable coli infection. The difficulty mentioned above of obtaining "normal" patients undoubtedly free from a coliform infection, was also referred to by these authors. Although these investigators only tested the sera of their "normal" cases against examples of typical *B. coli communis*, their results support very strongly the findings obtained by us in Kenya, more especially so as in our experience a considerable number of the coliform bacilli isolated from patients suffering from coli infections, conform in cultural reactions at least with the type *B. coli communis*. It seems fair to believe therefore that in the blood of "normal" persons, agglutinins for coliform bacilli do not exist except in inappreciable amounts, so that when they are detectable in 1 in 100 dilutions of the blood serum, they may be looked on as pathological and as evidence of a coli infection. The only objection which can be raised to this conclusion is that, as will be shown in a later section, coli agglutinins produced artificially are specific for the bacillus used as antigen, and therefore it may be argued that experiments conducted on "normal" cases with stock coli antigens are of no value. This objection however only holds good for the experiments of Dudgeon, Wordley and Bawtree, as the tests made by us were carried out with the full technique employed by us in making up autogenous vaccines of coliform bacilli and described in

<sup>1</sup> Dudgeon, Wordley and Bawtree (1921). *Journ. Hygiene*, xx. 149.

<sup>2</sup> Dudgeon, Wordley and Bawtree (1922-3). *Journ. Hygiene*, xxi. 178.

the second section of this communication. It may be pointed out here, as it will be again later, that the very specificity of coli agglutinins is evidence in favour of their being pathological when they are found in a patient's blood.

#### V. AGGLUTINATION REACTIONS FOR AUTOGENOUS COLIFORM BACILLI SHOWN BY "INFECTED" PERSONS.

As has been explained in a preceding section the standard demanded by us before we recognise that a person is suffering from an infection with one or more coliform bacilli, is the ability of that person's blood serum to agglutinate such coliform bacillus or bacilli in a dilution of 1 in 90. It is not uncommon however to find that the blood serum in these cases causes agglutination of coliform bacilli in dilutions several times greater than 1 in 90. The highest titre obtained has been one of 1 in 800, but titres of 1 in 160 and 1 in 320 have been comparatively common. Dudgeon, Wordley and Bawtree<sup>1</sup> found a titre of 1 in 2000 in one patient who was the victim of a coli infection. It is also not unusual in our experience to find that agglutinins are present in a patient's serum for two and sometimes three different coliform bacilli isolated from his faeces, though ordinarily not in identical amounts. A practical question then arises as to whether we are going to consider all these bacilli as infecting agents, or to conclude that the lower titre agglutinins are examples of secondary agglutinins produced in response to infections with the coliform bacillus, the agglutinin titre for which is highest. In view of the great specificity of agglutinin formation for coliform bacilli produced experimentally in rabbits, the second alternative would seem to be an improbable explanation, and we must then believe that these are cases of double infection with coliform bacilli, possibly at different periods, thus partially accounting for the differences seen in titre. This question is of the greatest importance in view of the fact that we have to set an artificial boundary between agglutinin titres which can be taken as evidence of infection with coliform bacilli and those which cannot, and it is essential that this boundary should not be too high for fear of excluding true cases of infection.

In regard to cross-agglutination between the sera of, and the coliform bacilli isolated from, different patients no results worth recording have been obtained up to the present. It has only been possible to apply this test in a few instances, and in only two cases was any evidence of cross-agglutination found. In all other cases the serum from one patient failed to cause agglutination of coliform bacilli from any of the other patients, even though by cultural reactions some of these bacilli were identical with those obtained from the patient whose serum was being tested. As will be described in a later section, experimentally produced agglutinins for coliform bacilli are apparently specific for the bacillus used as antigen during the immunising process, so cross-agglutination is probably the exception rather than the rule.

<sup>1</sup> Dudgeon, Wordley and Bawtree (1922-3). *Journ. Hygiene*, xxi. 178.

## VI. THE ISOLATION OF COLIFORM BACILLI FROM THE BLOOD OF PATIENTS SUFFERING FROM COLI INFECTIONS.

In seven of our cases, cultures of the blood of the patient were made in lactose bile-salt broth, in ordinary broth, and on blood-agar with negative results. In one case also a culture was made from the synovial fluid of the knee-joint, again with negative results.

## VII. THE PATHOGENICITY FOR RABBITS OF COLIFORM BACILLI ISOLATED FROM "INFECTED" PERSONS.

Two of the organisms isolated from a case of chronic rheumatism, *Bacillus* 681 L and *Bacillus* 681 N, were tested for their pathogenic action on rabbits. A live bouillon culture of *Bacillus* 681 L (a true *B. coli communis* by cultural reactions) was inoculated into the synovial cavity of the femoro-tibial articulation of Rabbit 59. There was a marked local reaction, heat and tumefaction of the joint resulting, associated with loss of function. Besides the synovitis, there was also considerable swelling of the periarticular tissues of the joint, although precautions were taken to ensure that the culture was injected into the synovial cavity only, and by the use of a very fine needle that leakage was prevented along the needle track after withdrawal. There was practically no alteration in the animal's temperature or weight, while, apart from its obvious disinclination to use the affected limb, the rabbit never appeared indisposed. A control injection of sterile bouillon was made into the corresponding joint of the opposite side of the animal, but no change other than a very slight synovitis causing no disability, was seen, and the leg was used quite freely. The same experiment was made on Rabbit 58, using this time *Bacillus* 681 N (a coliform *Bacillus* Type V), with similar results. A living culture of this same *Bacillus* 681 N was also injected intravenously into Rabbit 60 without causing any apparent indisposition, or any change in weight or temperature. It may also be recorded here that six rabbits used for the production of immune sera were inoculated intravenously on three occasions at weekly intervals with live cultures of six different types of coliform bacilli with no apparent ill-effect other than the production of a small abscess where leakage of the culture occurred from the vein into the surrounding subcutaneous tissue. Rabbit 29 was used as a control throughout. It is noteworthy that no suppuration occurred in either the joints or the periarticular tissues, and that the disability produced by the injection of the live cultures into joint cavities gradually disappeared, and the joints became clinically normal again.

The experiments recorded here cannot be considered to reproduce in the rabbits the state of constant absorption of bacillary toxins which we believe occurs in patients suffering from coli infections, and indeed they were only made as a preliminary step to form a guide as to the best experimental course to pursue in the investigation. Unfortunately a serious epidemic among the laboratory animals caused so great a shortage of rabbits that we were forced

to abandon the experiments for the time being, though it is hoped that they will be continued as soon as the supply of animals permits. From the few results recorded above, no conclusions of any value can be legitimately drawn.

# VIII. THE CLASSIFICATION OF THE COLIFORM BACILLI ISOLATED FROM PATIENTS SUFFERING FROM COLI INFECTIONS.

## A. CLASSIFICATION BY CULTURAL REACTIONS.

The cultural reactions of most of the coliform organisms isolated from patients in the course of the investigations described in this paper are given in Table II as far as they are known. In a few instances the cultures were lost before it was possible to examine them. Unless the reactions shown by a bacillus were typical of an already recognised and named member of the coli-typhoid group, they were re-tested, and in some cases where the variation from a known bacillus was small, this was done as many as four times.

Table II.

NOTE. A = Acid formed. G = Gas formed. sG = Slight gas formation. C = Clot formed. T = General turbidity. P = Pellicle formed. Alk. = Alkali formed. + = Positive. 0 = Negative. ± = May be positive or negative. ? = Not examined. \* = Name suggested for this organism.

Culture Group No.	No. of times isolated	No. of patients in which found	Cultural reactions found														Remarks	
			Lactose	Mannite	Glucose	Saccharose	Dulcete	Sallein	Adonite	Sorbite	Inosite	Litmus milk	Indol production	Voges-Proskauer reaction	Gelatin	Bouillon		Motility
I	49	39	AG	AG	AG	AG	AG	AG	0	AG	0	A → AC	+	0	0	TP	+	<i>B. pseudocoli</i>
II	5	5	AG	AG	AG	AG	AG	AG	0	AG	AG	A → AC	+	+	0	TP	+	
III	1	1	AG	AG	AG	AG	AG	AG	AG	AG	AG	A → AC	+	+	0	TP	+	
XXV	1	1	AG	AG	AG	AG	AG	AG	AG	AG	0	A → AC	+	+	0	T	+	
IV	19	17	AG	AG	AG	AG	AG	0	0	AG	0	A → AC	+	+	0	TP	+	<i>B. nairobiensis*</i>
V	13	12	AG	AG	AG	AG	0	0	0	AG	0	A → AC	+	0	0	T	+	<i>B. kenyaensis*</i>
VI	15	15	AG	AG	AG	AG	0	0	0	AG	0	A → AC	+	+	0	T	+	<i>B. pseudocoloides A.</i>
VII	3	3	AG	AG	AG	AG	0	AG	0	AG	0	A → AC	+	+	0	T	+	
VIII	1	1	AG	AG	AG	AG	0	AG	AG	AG	AG	A → AC	+	0	0	T	0	
IX	6	5	AG	AG	AG	AG	0	AG	AG	AG	0	A → AC	+	+	0	T	+	
X	4	4	AG	AG	AG	AG	0	AG	0	AG	AG	A → AC	+	+	0	T	+	
XI	4	4	AG	AG	AG	0	0	0	AG	AG	0	A → AC	+	+	0	T	+	<i>B. acidi lactici</i>
XII	15	13	AG	AG	AG	0	0	AG	0	AG	0	A → AC	+	0	0	TP	+	<i>B. paragrünthali</i>
XIII	16	16	AG	AG	AG	0	0	AG	AG	AG	0	A → AC	+	0	0	T	+	<i>B. ukambaensis*</i>
XIV	36	32	AG	AG	AG	0	AG	AG	0	AG	0	A → AC	+	0	0	T	+	<i>B. coli communis</i>
XV	10	10	AG	AG	AG	0	AG	0	0	AG	0	A → AC	+	0	0	T	+	<i>B. metacolooides</i>
XVI	3	3	AG	AG	AG	0	AG	0	0	AG	AG	A → AC	+	0	0	T	+	
XXVI	1	1	AsG	AG	AG	0	AG	AG	AG	0	0	A → AC	+	0	0	T	+	
XXVII	2	2	AG	AG	AG	0	0	0	0	AG	0	A → AC	+	0	0	T	+	
XXVIII	1	1	AG	AG	AG	0	AG	AG	0	0	AG	A → AC	+	0	0	T	+	
XXIX	2	2	0	A	A	A	0	A	0	A	Alk.	+	0	0	0	T	+	It is doubtful where these bacilli should be classified. As all were agglutinated by the patient's serum, they may be variants of coliform bacilli which have temporarily lost their power of fermentation
XXX	1	1	0	A	A	0	0	A	0	A	Alk.	+	0	0	0	T	+	
XXI	1	1	0	0	0	0	0	0	0	0	Alk.	0	0	0	0	T	+	
XXII	2	2	0 (or A)	A	A	0	A	0	0	A	0	A → AC	+	0	0	T	+	
XXIII	1	1	A	A	A	A	AG	A	0	AG	0	A → AC	+	0	0	T	+	
XXIV	1	1	0	A	A	A	0	A	0	0	Alk.	+	0	0	0	T	+	
XXV	1	1	AG	0	AG	0	0	AG	0	?	0	A → AC	+	0	0	T	+	
XXX	1	1	0	AG	AG	0	AG	0	0	?	?	A → AC	0	0	0	T	+	
XXX	1	1	0	0	AsG	0	0	0	0	?	?	Alk.	+	0	0	T	+	

A word is first necessary in regard to the culttral reactions chosen as a basis for identification. The classification of the group of coliform organisms is still in a state of chaos, more especially so when it is founded on the action of the bacilli on various carbohydrates and proteins. Papers are constantly

being published suggesting new chemical reactions as a basis for grouping these bacteria, but most of these have not so far received the general recognition of bacteriologists. MacConkey<sup>1</sup> suggested that lactose primarily, and saccharose and dulcitol secondarily, should be used to differentiate various classes of coliform bacilli. Another author has advised an amendment by the replacement of dulcitol by salicin as leading to a separation into groups which are better correlated with their natural sources than those obtained by MacConkey's suggestion. A new step in the separation of the coliform organisms into classes was taken by Clark and Lubs when they introduced the gas ratio obtained during fermentation by the different bacilli as a basis for differentiation. The same authors in a later paper<sup>2</sup> found that acid production, as shown by the methyl-red test, is correlated closely with the gas ratio if media of definite composition are used, and thus a simple test, applicable in any laboratory, is given to the bacteriologist. Levine<sup>3</sup> has shown that the methyl-red test and the Vosges-Proskauer reaction follow each other very closely, so that it is really unnecessary to apply both when studying the biology of a coliform bacillus. Incidentally it may be noted that Levine states in the same paper that coliform bacilli giving a positive Vosges-Proskauer reaction are rare in faeces, and that amongst the bacilli isolated from our patients with coli infections, under 10 per cent. have given a positive carbinol test. Mackie<sup>4</sup> believes that inositol is a substance, the fermentation or non-fermentation of which has considerable claims to being taken as a basis for differentiation. In choosing the cultural reactions, therefore, to which the coliform bacilli isolated from our cases were to be submitted, due consideration had to be given to the suggestions of these and other authors, and as far as was possible with the materials at command, all suggested reactions, with the exception of the estimation of the gas ratio and the methyl-red test, which were omitted as the Vosges-Proskauer reaction was included, were employed in our examination. The results have led to the conclusion that as yet no sound classification based on cultural characteristics has been evolved for the coliform bacilli. Certainly, as will be seen from Table II which records the cultural reactions of most of the bacilli isolated by us, a very considerable number fall into fairly large and well-represented groups, some of which agree with coliform bacilli already named, and some of which on the other hand have not received a designation, at least so far as the literature which is available to us shows. For one or two of these unnamed groups containing a number of our coliform bacilli provisional names have been suggested. On looking at Table II, one cannot but be struck by the number of groups represented by only one or two bacilli, which vary from already described and named types in one or two reactions. As previously stated such bacilli were

<sup>1</sup> MacConkey (1905). *Journ. Hygiene*, v. 333.

<sup>2</sup> Clark and Lubs (1915). *Journ. Infec. Dis.* xvii. 160.

<sup>3</sup> Levine (1916). *Journ. Infec. Dis.* xviii. 358.

<sup>4</sup> Mackie. *Applied Bacteriol.* p. 209.

re-tested as regards cultural reactions several times, but maintained the differences found at their initial examination. They must therefore be looked on as true variations from known species, but inasmuch as the variations shown are not limited to one particular reaction, but involve each of the cultural reactions used when the variants are taken as a whole, it is obvious that no reaction at present employed in the classification of coliform bacilli can as yet be considered entirely satisfactory. We have made attempts to produce variants of certain bacilli which do not ferment saccharose, mannite or lactose by growing such bacilli in media containing one or other of those sugars, and subculturing them daily. Success has been obtained with comparative ease in the case of lactose; indeed it is a common incident in our experience for a lactose-fermenting bacillus after cultivation for some time on agar, to show a non-lactose fermenting variant, which after a few subcultures in a medium containing lactose, regains completely its power of fermenting the sugar. As regards saccharose we have been successful in one instance in converting a non-fermenting bacillus into a fermenting one after three months' daily subculture. This experiment is being repeated to obtain confirmation of the result. In the case of mannite, a bacillus which produced acid but no gas in media containing this alcohol, failed to produce gas-forming variants after daily subcultivation for four months. An interesting fact observed by us is that in the case of a few of our coliform bacilli, the original cultural reactions have been lost after artificial cultivation for some time.

The question of the existence of haemolytic properties amongst the coliform bacilli isolated by us has not yet been investigated, though this will be done at an early date.

#### B. CLASSIFICATION BY SEROLOGICAL REACTIONS.

##### 1. *Production from Rabbits of Immune Sera for Coliform Bacilli.*

In order to carry out serological tests on coliform bacilli, it was necessary to produce immune sera. Six bacilli typical of the main cultural groups of coliform bacilli isolated in the course of these investigations were selected, and 24 hours old peptone broth cultures prepared. Half a cubic centimetre of the living culture was inoculated into the marginal vein of a rabbit's ear, a second injection of a similar amount was given a week later, and a third injection of double the quantity administered after the lapse of another week. Eight days after this last injection the rabbit was bled, and the serum removed after the blood had clotted. When the sera so obtained were tested for agglutinative capacity against the bacillus used as antigen, titres ranging from 1 in 16,000 to 1 in 32,000 or higher were obtained.

No ill effects from the intravenous injection of these living cultures into the rabbits were seen, if one excepts the occasional small abscess which sometimes followed a leakage of the culture from the vein into the surrounding subcutaneous tissue.

*2. Agglutination Reaction Experiments.*

The method used in these investigations into the agglutination reactions of coliform bacilli was that described by Dreyer<sup>1</sup>, but modified by incubating the tests in a water-bath at 55° C. for a period of five hours instead of four. It was found most convenient to read the results immediately after the tubes were removed from the water-bath, and again after standing overnight, a period of approximately 16 hours elapsing between the two readings. The necessary dilutions were made by the use of Donald's dropping pipettes. It is necessary to lay stress on two important details of Dreyer's technique: the cultures must be made in veal broth, and the emulsions of the bacilli used in the test must be very thin. Unless these two instructions are strictly carried out, either the agglutination may fail completely, or false agglutination may occur.

Using then this slightly modified technique, the dilutions of the immune rabbit sera, obtained as described above, which produced standard agglutination (taken as the highest dilution showing distinct flocculation without precipitation) of the homologous bacillus, were found to be 1 in 16,000, 1 in 32,000 or 1 in 64,000 according to the serum examined. As a preliminary to attempting the serological grouping of the various coliform bacilli, and to determining the amount of cross-agglutination that was obtainable with artificially-produced agglutinating sera, a selection of eight to ten other bacilli was made from each of the cultural groups to which the bacilli used for immunising the rabbits, as described in Section VIII B. 1, belonged. In each group the bacilli selected had the same cultural reactions, and were tested in one batch for agglutination with the serum prepared against the representative bacillus of the group, which was also included in the test. The results obtained were, for our purpose, extremely disappointing. In the case of each batch, although the titre for the representative bacillus reached that originally found, the highest titre for other bacilli of the group was at the most 1 in 3000, and in most instances was under 1 in 2000. When cross-agglutination between the groups was tested, titres up to 1 in 3000 were obtained against heterologous bacilli. Although the technique of the test was modified in various ways in the attempt to find some method whereby each group of bacilli could be differentiated, no change made any alteration to the results obtained previously. The possibility of this low agglutination titre being a general reaction separating off the coliform bacilli causing coli infections from others, was not lost sight of, but when two coliform organisms which were isolated by the technique described in Section II, but were not agglutinated by the patient's serum, were tested with one of the immune rabbit sera, and gave approximately the same agglutination titre as agglutinable coliform bacilli, the conclusion was reluctantly reached that the sera produced by the inoculation of rabbits with coliform bacilli were specific for

<sup>1</sup> *Med. Res. Council* (1920). Special Report Ser. No. 51, p. 128.



the bacilli used in the immunising process, and that no group-agglutinins could be produced, so that classification of the coliform bacilli by agglutination reactions was impossible. A similar conclusion was reached by Mackie<sup>1</sup> and other observers.

The protocol of one of the agglutination tests is given in Table III, and as this is representative of the results obtained with all the immune sera, it is unnecessary to publish the remainder.

Table III.  
*Agglutination Tests.*

Culture number	Serum 682 F. Dilutions												Serum control
	1 : 2,000	1 : 3,000	1 : 4,000	1 : 5,000	1 : 6,000	1 : 8,000	1 : 10,000	1 : 12,000	1 : 14,000	1 : 16,000	1 : 24,000	1 : 32,000	
682 F	<i>T</i>	<i>T</i>	<i>T</i>	<i>T</i>	<i>T</i>	<i>T</i>	<i>T</i> -	<i>T</i> -	<i>S</i> +	<i>S</i> +	<i>S</i> +	<i>S</i> -	0
315 C	<i>S</i> +	<i>S</i> -	<i>S</i> -	tr.	0	0	0	0	0	0	0	0	0
445	<i>S</i> +	tr.	0	0	0	0	0	0	0	0	0	0	0
471 A	tr.	tr.	0	0	0	0	0	0	0	0	0	0	0
577	tr.	0	0	0	0	0	0	0	0	0	0	0	0
585	0	0	0	0	0	0	0	0	0	0	0	0	0
586	tr.	0	0	0	0	0	0	0	0	0	0	0	0
600 A	0	0	tr.	0	0	0	0	0	0	0	0	0	0
693 A	0	0	0	0	0	0	0	0	0	0	0	0	0
764 G	<i>S</i> -	tr.	tr.	0	0	0	0	0	0	0	0	0	0
768 K	tr.	0	0	0	0	0	0	0	0	0	0	0	0

NOTE.—(1) The notation used to indicate the degree of agglutination is that of Dreyer. *T*=total agglutination. *S*=standard agglutination. tr.=trace of agglutination.  
(2) Bacillus 682 F was that used as antigen in the production of immune serum 682 F. Bacilli 764 G and 768 K were non-agglutinable coliform bacilli. The other bacilli all belong to the same group as Bacillus 682 F.

### 3. Complement Fixation Reaction Experiments.

When the agglutination tests with immune sera failed to afford a basis for the serological grouping of the coliform bacilli, the complement fixation reaction suggested itself as a possible means of achieving the same aim, especially as Mackie<sup>2</sup> indicated that he had been able to obtain a certain amount of group reaction among the members of the coli family by it. The first essential was to develop a technique, as from investigation of the literature available, every worker on the reaction with bacterial antigens seemed to have devised a method for himself, a serious difficulty in the way of obtaining comparable results. In the Nairobi Laboratory, the method employed in the Wassermann reaction is that designated Method IV in *Report No. 14* of the Medical Research Council. As this has yielded most satisfactory results with a minimum of trouble in our hands, essentially the same method was applied in the study of complement fixation amongst coliform bacilli. The antigen employed was an emulsion of the bacillus under investigation, and this was standardised by the opacity method to contain 4000 million bacilli per c.c.

<sup>1</sup> Mackie. *Applied Bacteriol.* p. 203.

<sup>2</sup> *Ibid.* p. 206.

After titration of the complement in the same way as for the Wassermann reaction, the anticomplementary power of the antigen was tested by making varying dilutions of the standardised bacterial emulsion and finding that one which just failed to deviate a minimal haemolytic dose of complement. The antigen was used in the test proper in half the strength obtained by this titration. The haemolytic system employed was the same as in the Wassermann reaction, namely, a mixture containing 5 M.H.D. anti-sheep cell amboceptor and a 3 per cent. suspension of sheep red blood corpuscles. In the test proper, varying dilutions of the serum under examination ranging from 1 in 10, 1 in 20, 1 in 30, etc. up to 1 in 80, 1 in 90, 1 in 100, were put up with each bacterial antigen, the complement being used in two strengths, 3 M.H.D. and 15 M.H.D. The quantities of diluted serum, diluted antigen, and diluted complement were all equal, and can be designated as one volume. After the mixing of the reagents in the tubes, the latter were allowed to stand for half-an-hour at room temperature, and were then incubated in a water-bath at 37° C. for another half hour. At the end of this time, one volume of the haemolytic system was added, and the tubes, after shaking, again incubated in the water-bath at 37° C. for half-an-hour, after which the results were read.

Unfortunately this test again failed to demonstrate any grouping amongst the coliform bacilli examined. The homologous bacillus in the case of each serum caused a considerable amount of complement fixation, a 1 in 60 dilution of the serum giving almost complete fixation of 3 M.H.D. of complement, and a 1 in 20 dilution a similar degree of fixation of 15 M.H.D. of complement. When bacilli belonging to the same group according to cultural reaction, were tested, a 1 in 10 dilution of the immune serum only gave a very slight fixation with 15 M.H.D. of complement, and failed to fix more than half 3 M.H.D. of complement as judged by the amount of haemolysis. Bacilli which belonged to other coli groups by cultural reactions, gave approximately the same amount of complement fixation as bacilli of the same cultural group, and when bacilli which gave no agglutination with patients' sera, and belonged culturally to groups outside those found in our tests, were examined, they behaved in the same way as the non-homologous bacilli. A repetition of the tests confirmed the original findings. As this method of testing complement fixation differed somewhat from that employed by Mackie<sup>1</sup>, the same antigens and antisera were re-tested by a technique founded on the somewhat meagre description given by him in his paper, and completed by reference to the description of Browning's method of performing the Wassermann reaction given in *Report No. 14* of the Medical Research Council, which seemed to be the basis on which Mackie's method rested. Even by this change of procedure we failed to get any semblance of grouping, which was the more surprising as we were dealing with many bacilli which belonged to the division of non-inosite-fermenting indol-producing gas-forming coliform organisms which, according to Mackie, fall into one group in complement fixation. Several

<sup>1</sup> Mackie. *Applied Bacteriol.* p. 206.

modifications of technique were made both in the direction of increased delicacy and in the opposite one of greater coarseness, but by no method was there any indication of grouping, and we had reluctantly to come to the conclusion that as with the agglutination test, so with the complement fixation test, the antigenic properties of the coliform bacilli were too specific to allow of a basis for grouping being found. The use of defatted coliform bacilli as antigens for the production of immune sera is under investigation, and it may prove to be the solution of the difficulty of obtaining group reactions.

A protocol of one of the complement fixation tests is appended in Table IV as an example of the results found by us. Those obtained with other immune sera and other coliform bacilli were identical, so that it is unnecessary to publish all.

Table IV.

*Complement Fixation Tests.*

Dosage of complement	Culture No.	Serum 682 F. Dilutions										Serum control without antigen
		1:10	1:20	1:30	1:40	1:50	1:60	1:70	1:80	1:90	1:100	
3 M.H.D.	682 F	0	0	0	0	+	+	++	++	++	++	++++
	315 C	++	++	+++	+++	++++	++++	++++	++++	++++	++++	++++
	445	++	++	+++	+++	++++	++++	++++	++++	++++	++++	++++
	471 A	+++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	577	++	++	+++	+++	++++	++++	++++	++++	++++	++++	++++
	585	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	586	++++	++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	600 A	++	++	+++	+++	++++	++++	++++	++++	++++	++++	++++
	693 A	++	++	+++	+++	++++	++++	++++	++++	++++	++++	++++
	764 G	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
15 M.H.D.	768 K	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	682 F	0	+	++	++	++	+++	+++	+++	+++	+++	+++
	315 C	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	445	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	471 A	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	577	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	585	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	586	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	600 A	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	693 A	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	764 G	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	768 K	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

NOTE. (1) The degree of haemolysis is expressed by the number of crosses. 0 = No haemolysis. +++ = Complete haemolysis.

(2) Bacillus 682 F was that used as antigen in the production of serum 682 F. Bacilli 764 G and 768 K were non-agglutinable coliform bacilli. The other bacilli all belonged to the same group as Bacillus 682 F.

#### 4. *Precipitin and Bacteriolytic Reaction Experiments.*

As agglutination and complement fixation tests have failed to give a serological basis of classification of the coliform bacilli, the precipitin and bacteriolytic reactions are in the process of being examined for the purpose. So far no results are to hand, but it is hoped to furnish a report on them at a later date. The experience of Dudgeon, Wordley and Bawtree<sup>1</sup> with precipitin tests applied to coli strains isolated from the urine would suggest however that these are as specific as those of complement fixation.

<sup>1</sup> Dudgeon, Wordley and Bawtree (1922-3). *Journ Hygiene*, xxi. 192.

*5. Saturation Experiments.*

Strictly speaking our immune sera should have been subjected to desaturation experiments, but we felt that owing to the specificity found in our antisera for their respective antigens, and the almost complete absence of interaction with heterologous antigens, it was unnecessary to carry out these tests, at any rate while so many and more hopeful lines of examination remained open to us.

From the results reported above on the experimental investigation into the classification of coliform bacilli isolated from our patients, it can only be concluded that up to the present, serological reactions have failed to help us, indeed they have increased our difficulties by suggesting that antigenic differences exist even amongst bacilli which fall into the same group according to cultural reactions. On the other hand, the very specificity of the agglutinins in an artificial immune serum for the homologous bacillus, strongly supports our view that the agglutinins for coliform organisms which were found in the blood of our patients were not accidental, but the result of infection.

## IX. DISCUSSION.

For long the subject of toxic absorption from the bowel has been discussed in medicine, and the results of vaccine treatment recorded above support the views of those who hold that this absorption is responsible for much disease, and at the same time afford an explanation of the cases of failure which have occurred, with remedies directed against toxic absorption generally, and not against a specific infective agent.

It will have been obvious from all that has been said in the preceding sections that the clinical use of our vaccines is based on a theory that if a patient has in his blood a certain concentration of agglutinins for a coliform bacillus isolated from his faeces, he is suffering from an infection with, or from the absorption of toxins elaborated by, that coliform bacillus. In this section it is our object to set out the facts noted clinically or experimentally, which are or are not in accordance with this theory.

In the first place we have to consider the results of the examination of "normal" persons for agglutinins for coliform bacilli. It is usually held that the presence of agglutinins in the blood in any quantity is the result of an attempt on the part of the body to produce immunity to the organism agglutinated or to one of its near allies. It is however recognised that agglutinins for certain bacteria may be present normally in the blood of certain individuals, in some cases in comparatively high concentrations. In the case of the coliform bacilli we are met with an initial difficulty in that it is often almost impossible to be certain whether a person is free from a coli infection or not, as so many persons who believe themselves healthy and only occasionally suffer twinges of "rheumatism," present on examination the symptoms of an infection with one or other member of the coli group. Such cases must be carefully eliminated

from any series of "normal" persons who are being examined for the presence of "natural" agglutinins for coliform bacilli. As stated above, 54 apparently "normal" cases have been examined by us, and so far as a careful clinical examination revealed, none of them showed any evidence of a coli infection. Of this series the blood serum of only two individuals had an agglutination titre of 1 in 90 for coliform bacilli isolated from their faeces. This result is supported by the work of Dudgeon, Wordley and Bawtree who report that in one series<sup>1</sup> of 66 "normal" cases, the serum of five only agglutinated a stock *B. coli communis* in a dilution of 1 in 50 (Dreyer's method), while in another series<sup>2</sup> of 104 cases, the serum of 22 only had an agglutinin titre of 1 in 50 for *B. coli* antigens, two of these patients later being found to have a probable coli infection. These results contrast markedly with those obtained in the cases set out in Table I, for the serum of all these showed the presence of agglutinins for coliform bacilli isolated from their faeces in a dilution of at least 1 in 90, and in many instances in dilutions varying from 1 in 160 to 1 in 800. It is important to note also that, whereas Dudgeon, Wordley and Bawtree have only tested their sera for agglutination with typical strains of *B. coli communis*, an organism always present in the faeces, many of our patients have shown agglutination in high dilutions for atypical coliform organisms, a very significant fact taking into consideration the marked specificity of agglutinins for members of the coli group recorded in the preceding section, and the dictum of Robertson<sup>3</sup> that "Aberrant types of *B. coli communis* are somewhat common, especially in the intestine, and there are very good grounds for believing that they are in nearly every instance in which they occur exercising a pathogenic action."

Certainly it is very noticeable that when plated out on neutral-red bile-salt lactose peptone agar, the faeces of patients showing agglutinins for coliform bacilli in their blood, usually give a much larger variety of colonies than those of persons who do not possess such agglutinins.

The somewhat meagre evidence afforded by injections of live cultures of coliform bacilli into joints, if anything, supports the belief that the coliform bacilli isolated from our cases have a selective pathogenic action, since inflammatory changes without suppuration were produced mainly in the peri-articular tissues, and only to a small extent in the synovial membrane, and soon subsided, leaving a clinically normal joint. The experiments in this direction so far are too few in number to justify definite conclusions, and must be repeated and extended to form the subject of another report at a later date.

So far the evidence we have put forward has been based on results obtained by laboratory methods rather than on clinical facts observed in our patients, and we must now pass on to this new line of evidence, though first making a slight digression in order to clarify our ideas. The summary which is found

<sup>1</sup> Dudgeon, Wordley and Bawtree (1921). *Journ. Hygiene*, xx, 149.

<sup>2</sup> Dudgeon, Wordley and Bawtree (1922-3). *Journ. Hygiene*, xxi, 178

<sup>3</sup> Robertson. *Therapeutic Immunization*, p. 143.

at the foot of Table I is liable to give the impression that we have been treating widely different clinical conditions with our vaccines, but although the familiar labels for diseases may suggest this, a careful consideration of the typical symptoms exhibited in those diseases will show that a toxæmia might well explain them all, the variations exhibited from patient to patient which lead to the emphasis of different symptoms and physical signs and so to a different diagnostic label, being the result of idiosyncrasy on the part of the different tissues, or possibly the effect of local injury. In support of this latter suggestion we may quote the work of Brewer<sup>1</sup> who showed that if the lumbar region of an animal is bruised and living cultures of bacteria are injected into the vein of its ear, an acute surgical kidney will develop, whereas when no trauma has occurred, the kidneys remain healthy. Now the general symptoms of a coli toxæmia have been recognised fairly widely for a long time, general malaise and "rheumatic" pains being two of the most prominent ones. If then we find an agglutinin for a coliform bacillus in the blood of a patient exhibiting these symptoms, we are justified in suggesting that there is probably a causal connection between that coliform bacillus, and the disease. The belief that the symptoms we are referring to are probably the result of a coli infection, receives support from an incident recorded by Savage<sup>2</sup> who quotes it from a statement of the Vaughans. These latter investigators

worked with large masses of *B. coli* grown for two weeks in agar in metal tanks. At the end of that time the crude bacterial substance obtained by extracting this mass with absolute alcohol and ether was powdered finely in an agate mortar for certain special investigations. They state "the person who did the pulverizing was often quite seriously poisoned during the process unless he took the precaution of wearing a mask which hindered the inhalation of the powder." Apart from symptoms due to direct irritation on the mucous membrane the chief symptoms were a feeling of depression and malaise and a chilly sensation. Occasionally a decided chill would be experienced, but no temperature readings were taken. Nausea and even vomiting were occasionally noted. After a period of discomfort varying from six to ten hours, during which the patient often complained of dull pains in the various joints, recovery would rapidly and completely take place.

It is inevitable that when a disease is the result of an attack on the connective tissues by bacterial toxins, the symptoms should be varied. Indeed, if the literature which has arisen on the subject of rheumatism is read, we cannot but be struck by the great variety of symptoms shown by diseases described by different authors under the same name, but careful consideration of each case shows that there is a marked resemblance between the general symptoms and the local symptoms, if due regard be paid to the severity of the infection and to the sites attacked, and allowance made for variations of resistance shown to bacterial toxins by different individuals.

To return now to clinical facts observed in our patients, attention must be drawn to the cases recorded in an earlier section of this paper. In Case 32,

<sup>1</sup> Brewer. *Surg. Gynaec. Obst.* II. 485; *Journ. Amer. Med. Assoc.* LVII. 179.

<sup>2</sup> Savage. *Food Poisoning and Food Infection*, p. 98.

we have an illustration of the effect of a vaccine made from an atypical coliform bacillus on a patient suffering from inflammation of an old gunshot wound of the leg, which was known to have been infected originally with a coliform organism. Here we know that an infection of the patient's tissues with a coliform bacillus had taken place through a gunshot wound five years before, that the wound after healing was liable to recurrent attacks of acute inflammation, that the patient's blood when examined five years after the original infection contained an agglutinin for an atypical coliform bacillus present in the patient's faeces, and finally that the inflammatory condition in the leg completely subsided under treatment with a vaccine prepared from this atypical coliform bacillus. These facts seem to point to the conclusion that the agglutinins found in the patient's blood were not "natural," but were a direct result of infection with a coliform bacillus at the time of the original wound, and that their persistence for so long a period as five years showed that an infection was still present in the patient's tissues. In Case 38 we see that from the faeces and urine of a patient, the same atypical coliform bacillus was isolated, showing a definite infection of the tissues of the patient, whilst agglutinins for this bacillus were found in the patient's blood serum. This is of importance as evidence that an atypical coliform bacillus in the faeces can produce an infection, which leads to the formation of agglutinins in the blood.

A still more interesting fact is the change of type of the coliform bacillus in those cases in which a relapse has occurred, which is explicable on the ground that where the body has been immunised against a coliform bacillus, this is able to produce variants which, owing to the strictly specific antibodies apparently produced with this group of organisms, are able to carry on the disease.

Finally, in setting out the evidence in favour of our theory, we cannot neglect that afforded by the results of vaccine treatment based on our ideas. A success rate as high as our figures show is strong evidence of the value of the vaccines, and it is difficult to explain it on any grounds other than that we have contrived to incorporate in them the causative organism of the disease. It is hardly conceivable that the effects of the vaccines are due to non-specific protein administration, for if they were, a stock vaccine should produce the same results as an autogenous one whereas we know by experience that it does not. In the early days of the vaccines, realising that so many different and often unrecognised factors enter into the cure of a disease, we hesitated somewhat in rushing to claim a high curative value for our method of treatment, but now that over 100 patients have been or are under observation, a total of 56 cases (out of 70 whose treatment has been completed) in whom a cure or great improvement has been wrought, justifies us in believing that our theory regarding agglutinins for coliform bacilli is correct, and that treatment based on that theory offers a very great hope of cure or alleviation in diseases in which such agglutinins are found in the patient's

blood. A less reliable but still a significant indication of the value of the vaccines is afforded by the number of cases sent to us by former patients.

This completes the evidence we have to offer in support of our theory regarding the presence of agglutinins for coliform bacilli in a patient's blood. Against our ideas it may be urged that a suspiciously large proportion of our patients have given positive agglutination results, but in opposition to this it must be stated that a number of persons whose cases are not recorded here, have been examined with negative results in spite of their showing signs indicative of a coli infection. Further indeed we may say that in view of the fact that all our patients approach us for the relief of a definite group of symptoms, it would be strange if we did not have a large proportion of positive results, unless our theory were wrong.

Another objection which can be raised against the matter contained in this paper is that we are basing our conclusions on the results obtained from a very small number of patients, who show a large variety of symptoms. Naturally in a country like Kenya which has a very small and very scattered white population, it is not to be expected that we should be able to show the numbers that could be obtained in England, and allowing for this a record of 70 completed cases is not to be despised as evidence, especially as many of the patients have been under observation for three or more years after treatment ended. As to the symptoms being so varied, this point has been dealt with somewhat at length above.

It must be granted as an objection to our theory that blood culture has so far given negative results, but it must also be remembered that it has only been possible to carry it out in a comparatively small number of cases, none of these being in the acute stage. It is also quite possible, indeed very probable, that in these patients we are dealing rather with a toxæmia than a bacteraemia, especially as Dudgeon, Wordley and Bawtree<sup>1</sup> have shown that the injection of filtered broth cultures of coliform bacilli can give rise to agglutinin production in rabbits.

A further objection, which could be based on our experimental results, is that the injection of live cultures of coliform bacilli intravenously in rabbits gave rise to no other ill-effects than the formation of local abscesses. (It will be noted that strangely enough the leakage of live cultures of coliform bacilli into the subcutaneous tissue of a rabbit's ear causes the formation of a local abscess, whereas the direct injection of such cultures into a joint causes no suppuration.) This is true, but in these animals only three injections were given and these at weekly intervals, whereas the probable condition for the production of disease by coliform bacilli is the constant slow absorption of the bacterial toxins, coupled with some injury such as results from a strain to the connective tissues of a part. Brewer's work<sup>2</sup> already quoted, supports this view. It may not even be necessary to have an injury in the case of a

<sup>1</sup> Dudgeon, Wordley and Bawtree (1922-3). *Journ. Hygiene*, xxi. 184.

<sup>2</sup> Brewer. *Surg. Gynaec. Obst.* II. 485; *Journ. Amer. Med. Assoc.* LVII. 179.



coli infection, but merely a state in which the normal tissue changes of a part have become unhealthy owing to a general lowering of vitality.

Weighing up now the evidence that has been set out, it can only be concluded that the balance of it is in favour of our contention that the presence of agglutinins for a coliform bacillus in the blood is evidence of a pathological condition resulting from the infection of certain of the tissues of the body with that bacillus, and that vaccines made from this presumed causative organism exercise a marked curative effect in these cases. We do not presume to say that vaccines afford the only means of cure, nor even that they are the best means, but we do believe that they have consistently given better results than any other method of treatment so far reported. At the present moment when we are hearing of the cure of sprue cases by the administration of parathyroid extract<sup>1</sup>, we have felt it necessary to investigate our cases, more particularly those which have shown recurrences, with a view to determining the amount of combined and free calcium present in their blood serum, and we are also testing the effects of parathyroid extract alone and in combination with the coli vaccine. If parathyroid extract is all that is necessary, we should expect that the agglutinin content of the blood would disappear under its exhibition. This, however, is matter for a later report, and is only mentioned to indicate the lines along which we are now working. Should parathyroid extract prove to be the only essential curative agent, the initial steps of our technique for the preparation of the vaccine will still be necessary to show the presence or absence of agglutinins.

A few words are necessary to indicate some of our views on certain aspects of the pathology of the infection. Fortunately or unfortunately no post-mortem examinations have been possible on any of our patients as none have died, and most unfortunately owing to the necessity of preserving our laboratory animals, we have not been able to spare a rabbit to see the pathological effects of an experimental coliform infection. Under these conditions, naturally, we are unable to describe any pathological histology, and we are entirely in the dark as to whether the tissue changes seen in the various cases are identical or not.

Since it seems likely that we are dealing with a toxæmia, or at most a bacterial infection producing its pathogenic effects by its toxins, it is probable that similar symptoms can be brought about by other bacterial toxins, so that our results are not adverse to the theory that pyorrhoea is a cause of rheumatoid arthritis, but rather support and extend that theory, suggesting indeed the application of our technique to the organisms isolated from the mouth to see if agglutinins are also formed in the blood for any of them.

The question of the site of origin of the bacteriaemia or toxæmia is a difficult one to decide with our present scanty knowledge. Undoubtedly chronic constipation is a very marked feature in the symptomology of the diseases we have treated, but whether as cause or effect has been impossible

<sup>1</sup> Scott (1924). *Brit. Med. Journ.* II. 305 (contains references as well to his former papers).

to determine. The experiments of Thiele and Embleton who painted *B. coli* on the glans penis or anterior portion of the urethra of a rabbit, and recovered them later from the iliac and renal lymph glands, and eventually from the urine, suggests that coliform bacilli have no difficulty in penetrating mucous membrane and in reaching the blood stream. This may take place more readily in the unhealthy condition of the bowel induced by chronic constipation, or when an injured mucous membrane is left following diseases such as dysentery and colitis, though, as has been mentioned above, no proof exists at present of a bacteriaemia other than the finding of the same atypical coliform bacillus in both urine and faeces, and agglutinins for that bacillus in the patient's blood. The difficulty of postulating the penetration of the mucous membrane of the bowel by *B. coli* in chronic constipation and its apparent absence in health might be met with by an application of Besredka's local vaccination theory of typhoid immunity, and this might explain the cases which relapsed in our series, these being extremely difficult to account for on a theory of absorption of toxin from the bowel. However, as can be seen from the above statements, we have no solid facts on which to form a hypothesis as yet, and until they can be produced, a discussion of the possible pathology of the disease is of but little practical use.

Finally in this section some reference must be made to our attempts to classify the coliform bacilli isolated by us. The results of our investigation have been to show that the coliform bacilli obtained from our patients cannot be grouped by any of the cultural reactions which have been suggested from time to time, without the production of a large number of groups containing only one to three representatives, all of which are probably variants of the larger groups. As these variations are not limited to one reaction, but involve all the reactions used when the variants are considered as a whole, it is obvious that it is impossible to say where variation begins and ends. It is stated that certain cultural reactions are stable for particular types of bacilli, but our experience would suggest that this may be so on artificial media but not for bacteria living in the body. Experiments indeed are proceeding to see whether it is possible to produce variants of coliform bacilli by altering the diet of the animal. As regards the types of coliform bacilli that we have isolated in the examination of our patients, it may be surmised from consideration of the number of times certain of them have been found in association and agglutinated by the same patient's serum, that the following groups in Table II are really identical, representing merely accidental variants from one another. These are Groups I, II, IV, V, VI, VII, IX and XIII; Groups XV and XXVII; Groups XII and XVII. It is possible also that Groups I, XII, XIV and XV are also really identical, and if this is proved later to be so, then the present division of coliform bacilli would not be justified, and it would probably be found that this group of organisms could be divided into two or three sections, in each of which the variants from type would be innumerable. If some such discovery is not made, it seems probable that the multiplication of species

in the group of coliform bacilli will go on until the possible number of combinations afforded by the cultural reactions which can be applied, is exhausted.

An interesting problem is opened up by the existence of these variants. Several of our patients who suffered from relapses during the course of their treatment, when re-tested for the presence of agglutinable coliform bacilli in their faeces, yielded a different organism culturally from that originally isolated, though usually the differences were only shown in one or two reactions. A possible explanation of this phenomenon is that variants of the original infecting bacillus have been produced which having different antigenic factors, escape the action of the immune substances produced by the body against the original bacillus as a result of the administration of vaccine. Knowing the marked specificity of the serological reactions of the coliform bacilli as seen *in vitro*, this explanation must be considered a possible, if not indeed a probable one. In connection with this, should the experiments in hand at present for the production of variants from type coliform bacilli be successful, it will be necessary to test these artificially produced variants as regards their antigenic similarity to or dissimilarity from the parent organism. If one does not admit the feasibility of this antigenic change, another possible explanation of the alteration of type of the infecting bacillus in the relapses of our patients, is that these variants were present in the patient's faeces from the start, and that when the body became protected against the original infecting bacillus, these variants got the opportunity of entering the tissues by the same portal as the first invading organism and, becoming pathogenic, caused a recurrence of symptoms. This explanation does not sound so convincing as the other as it is difficult to see why the variants should not have entered with the original infecting bacillus. A third explanation may just be indicated, namely that these variant bacilli arose on account of a change in the constitution of the faeces, and not as a result of the attempt of the original infecting bacillus to defeat the immune-bodies produced in response to the administration of the vaccine.

As regards serological classification of the coliform bacilli, we have to report that with agglutination and complement fixation tests, the immune sera proved so specific that no group reaction could be obtained. Possibly the use of defatted antigens may supply the necessary key to the solution of this difficulty.

#### X. SUMMARY.

A technique is described for the preparation of a vaccine from coliform bacilli isolated from the faeces of a patient and agglutinated by his blood serum.

The encouraging results obtained in the treatment of a number of patients suffering from rheumatoid arthritis, chronic rheumatism, and allied diseases with an autogenous vaccine prepared by this technique, are reported.

The absence of agglutinins for coliform bacilli from the blood of healthy persons is established, while it is shown that such agglutinins are present in high concentration in the group of diseases under consideration.

A report is made on the result of attempts to classify the coliform bacilli by cultural and serological reactions.

The facts reported are discussed, and the proposition adduced therefrom that if a patient has in his blood a certain concentration of agglutinins for a coliform bacillus isolated from his faeces, he is suffering from an infection with, or from the absorption of toxins elaborated by, that coliform bacillus, and a vaccine prepared from this organism will probably have a marked curative effect.

Suggestions are made as to the mechanism of infection with coliform bacilli, and as to the significance of variants in that group of organisms found in the faeces.

I have to acknowledge my indebtedness to Dr Gilks, the Principal Medical Officer, Kenya Colony, for permission to publish this paper, and for the notes on two of the cases; to my colleagues in the Laboratory, Dr Clearkin, Dr Allen, Mr Bailey and Mr Ramji Das, for suggestions and assistance in the preparation of the vaccines, for the obtaining of notes on many of the cases, and for the carrying out of the treatment in several instances; to Dr Welch and Dr Anderson for notes on some of the patients; and finally to Dr Ross who has not only provided notes on certain cases, but also obtained for me the 54 "normal" persons referred to in Section IV.

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## IMMUNISATION AND SELECTION AS FACTORS IN HERD-RESISTANCE<sup>1</sup>

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In considering the factors which are concerned in the spread of any epidemic bacterial infection among any host-population, the resistance brought into play by the latter will clearly be of crucial importance.

It has been suggested (Topley and Wilson, 1923) that the general problems of immunity and resistance have been studied too exclusively from the point of view of the individual, and that their reconsideration from the point of view of the herd would probably yield results of some interest. It is clear, for instance, that the herd possesses at least one method of acquiring resistance that is not at the disposal of the individual. If natural resistance be in any degree a variable character, the average herd resistance will be augmented by a simple sorting process during the spread of any epidemic infection associated with specific deaths among the population at risk, quite apart from any possible increase in the resistance of surviving individuals within that population.

As regards the spread of enteric infection among mice, we have learned that the survivors left after the passage of one epidemic wave show relatively high resistance, when exposed along with fresh susceptibles, to the risk of a second outbreak of the same disease. The level of resistance reached, however, affords no complete protection against a subsequent fatal infection. Although the survivors from the first wave tend to escape death during the early phases of the second epidemic, many of them succumb during its later stages (Topley, 1921; Amoss, 1922). Have these survivors acquired resistance during their exposure to the risk of infection, or do they merely represent that quota of the original population whose resistance, from the start, has been at a high level?

That variations in resistance to any given bacterial parasite do in fact occur among mice, as among all other animal species, has long been accepted, partly on the ground of actual observation, and largely on the ground of *a priori* probability. We owe to Webster the clear demonstration that, in the case of mouse typhoid, such variations are significant in degree.

Natural selection, then, will be provided with material susceptible to its inherent sorting action. It will certainly be operative during any one epidemic of disease, and will certainly tend to raise the average resistance to that

<sup>1</sup> A report to the Medical Research Council.

disease of the population at risk, provided that fresh susceptibles do not gain admittance during the epidemic period. Can we decide with any certainty whether or no, in the particular case of mouse typhoid, the acquirement of increased resistance by individual mice plays an additional part?

#### DATA ALREADY AVAILABLE.

Some light on this question may be gained by a study of the attempts which have been made to immunise mice against organisms of the enteric group, and especially against *B. aertrycke* (mutton), which is the chief cause of enteric infection among these animals. If active immunisation of the mouse against *B. aertrycke* were an easy procedure, attended with but slight mortality arising directly from the administration of the bacterial antigen, and resulting in a high degree of resistance, it would be reasonable to attribute to the development of an acquired immunity, during the actual spread of infection, an important share in bringing the epidemic to a close. If, on the other hand, it be found that such immunisation is difficult to carry out, often leading by itself to a high mortality among the treated mice, and that, even so, the resistance acquired is uncertain in its incidence and relatively slight in degree, then we should hesitate to believe an acquired immunity of this kind to be an important factor in checking the natural spread of disease.

It may be stated at once that, while certain of the earlier attempts at active immunisation against mouse typhoid yielded some degree of success (Loeffler, 1906; Wolf, 1908; Yoshida, 1909), the preliminary treatment was in most cases so severe as to result in a very considerable mortality, in some cases amounting to 50 per cent. of the treated animals. Within the last few years a growing realisation of the importance of studying the response of laboratory animals to those infections to which they are naturally liable, rather than their reaction to the injection, directly into their tissues, of bacteria which, so far as we know, do not attack them under natural conditions, has led to a somewhat extensive study of mouse typhoid in this country, in America and in Germany.

In Tables I to V<sup>1</sup> are summarised such of the experimental data available as bear directly on the question at issue. Many of these figures have been collected, in a somewhat similar form, by Neufeld (1924) whose general discussion deals with many of the fundamental problems of active immunisation.

It is not, we think, necessary to deal at any length with the data tabulated. It is clear that some degree of increased resistance against intraperitoneal or subcutaneous infection can be produced by parenteral administration of killed bacteria, in doses not of themselves lethal (Table I), and that this resistance is increased by increasing the number of preparatory inoculations (Webster, *i*, *k* and *m*). This increase in resistance is, however, relatively slight in degree, and is evidenced rather by a lengthening of the average time to death after

<sup>1</sup> The doses given in these tables have in all cases been calculated, as nearly as the protocols allow, in terms of the number of bacteria injected.

**Table I.**  
*Immunisation against B. aertrycke with dead cultures administered subcutaneously.  
Tested by intraperitoneal or subcutaneous inoculation.*

Series	Method of immunisation				Method of testing immunity				Mortality radio. Controls: immunised	Average time to death in days: immunised controls				
	Living or dead bacteria	Route of administration	Dose in bacilli	No. of times administered	Mortality % from immunisation	Route of administration	Dose in bacilli	No. of mice tested						
Webster (t) <i>Journ. Expt. Med.</i> , xxxvi, 83	D.	Sc.	2.5 × 10 <sup>8</sup>	1	0	Ip.	5 × 10 <sup>8</sup>	10	100	2	100	1	8	2.5
Webster (h) <i>Ibid.</i> , xxxvi, 84	D.	Sc.	2.5 × 10 <sup>8</sup>	2	0?	Ip.	5 × 10 <sup>8</sup>	20	90	3	100	1.1	10	2.6
Webster (m) <i>Ibid.</i> , xxxvi, 86	D.	Sc.	2.5 × 10 <sup>8</sup>	3	0?	Ip.	5 × 10 <sup>8</sup>	20	70	3	100	1.4	15.1	1
Neufeld (a) <i>Zeitsch. f. Hyg.</i> , cr. 472	D.	Sc.	2 × 10 <sup>8</sup>	1	0	Sc.	10 <sup>6</sup> to 10 <sup>10</sup>	11	100	5	100	1	5.8	1.8
Neufeld (b) <i>Ibid.</i> , cr. 473	D.	Sc.	10 <sup>8</sup> and 2 × 10 <sup>8</sup>	2	0	Sc.	10 <sup>4</sup> to 10 <sup>10</sup>	10	90	11	100	1.1	8.5	3.2
Lange and Yoshioka (f) <i>Ibid.</i> , cr. 461	D.	Sc.	1.3 × 10 <sup>10</sup> in all	8	0	Sc.	10 <sup>8</sup>	3	100	2	100	1	—	—
Lange and Yoshioka (h) <i>Ibid.</i> , cr. 461	D.	Sc.	1.75 × 10 <sup>10</sup> in all	8	33	Sc.	10 <sup>8</sup>	2	0	?	100	∞	—	—

the test inoculation, than by any marked increase in the proportion of ultimate survivors.

It appears (Table II) that inoculation of such killed bacterial antigens yields some definite protection against infection per os, as tested by intrastomachal injection (Webster, *j*, *l* and *n*), feeding with a dropping pipette (Lange and Yoshioka, *g*, *j*, *k* and *l*), feeding on bread soaked in culture (Topley and Wilson, *a*) or simple exposure to the risk of infection from other infected mice (Topley and Wilson, *b*). In the experiments recorded in this table there is not the same lengthening of the average time to death when the immune mice are compared with the controls, as was observable when the test dose was administered intraperitoneally or subcutaneously. It appears, in both tables, that the results obtained in this country and in America have yielded a far higher proportion of successes, as regards immunisation, than have been met with by Neufeld and his collaborators. This may, in part, be due to a difference in the pathogenicity of the strains of *B. aertrycke* employed. It may, in part, be due to the fact that, since relatively smaller numbers of mice were employed by the German workers, it was necessary to ensure the death of the controls, rather than rely on the comparative mortalities in the test and control groups, and hence relatively large test doses of living bacteria had to be employed.

Table IV shows that oral administration of living bacteria may lead to the acquirement of a relatively high degree of immunity to subsequent inoculations of living bacteria into the tissues, and that there is some association between a high mortality during the period of immunisation and the degree of resistance ultimately obtained (compare Webster, *c*, *f* and *h* with each other and with Lange and Yoshioka, *e*).

Table V shows that the same method of treatment results in a definite increase in resistance to subsequent administration of living *B. aertrycke* per os. The disparity between the results obtained at the Rockefeller Institute (Webster, *d*, *e* and *g*) and those reported from the Robert Koch Institute (Lange and Yoshioka, *a*, *b*, *c* and *d*) are here very marked.

#### EXPERIMENT.

In connection with another experiment, not yet reported, we desired to produce faecal excretion of *B. aertrycke* in a large number of mice. For this purpose 185 mice were fed, on several occasions during a period of one month, with broth cultures of this organism. At each feeding 0.02 c.c. of a 1/10 dilution of an 18 hours' broth culture, grown at 22° C., was administered per os to each mouse with a graduated dropping pipette.

One month after the last feeding the position was as follows. Of the 185 mice, 92 had died, in most cases from typical *B. aertrycke* infection. Of the remaining 93, 61 had excreted *B. aertrycke* on one or more occasions. The remaining 32 mice had never yielded cultures of this organism from the faeces, though specimens from each mouse had been repeatedly examined.



Table II.

*Immunisation against B. aertrycke with dead cultures administered subcutaneously or intraperitoneally.  
Tested by administration per os or by exposure to risk of infection.*

Series	Method of immunisation			Method of testing immunity			No. of control mice in test	Mortality % in controls	Mortality ratio. Controls: immunised	Average time to death in days: immunised	Average time to death in days: controls
	Living or dead bacteria	Route of administration	No. of times administered	Mortality % from immunisation	Route of administration	Dose in bacilli					
Webster (j) <i>Journ. Exp. Med.</i> xxxvi. 88	D.	Sc.	1	0	P.O.	$5 \times 10^8$	10	100	1	16.5	14
Webster (l) <i>Ibid.</i> xxxvi. 84	D.	Sc.	2	0?	P.O.	$5 \times 10^8$	20	55	100	120	14
Webster (n) <i>Ibid.</i> xxxvi. 86	D.	Sc.	3	0?	P.O.	$5 \times 10^8$	20	40	66.7	17	20
Lange and Yoshioka (g) <i>Zeitsch. f. Hyg.</i> ci. 461	D.	Sc.	8	0	P.O.	$10^{10}$	3	100	—	—	—
Lange and Yoshioka (j) <i>Ibid.</i> ci. 461	D.	Sc.	8	33.3	P.O.	$10^{10}$	2	50	100	2	—
Lange and Yoshioka (k) <i>Ibid.</i> ci. 461	D.	Sc.	11	82	P.O.	$10^{10}$	2	50	80	1.6	—
Lange and Yoshioka (l) <i>Ibid.</i> ci. 461	D.	Sc.	6	66.7	P.O.	$10^{10}$	4	100	—	—	—
Ornstein (b) <i>See Zeitsch. f. Hyg.</i> ci. 476	D.	Sc.	4	40	P.O.	$10^{10}$	6	100	100	—	—
Topley and Wilson (a)	D.	Ip.	2	26	F. 3 times and E.	?	90	54.4	88.9	1.6	—
Topley and Wilson (b)	D.	Ip.	2	31.8	E.	?	75	8	41.3	5.2	—

\*=In this and all tables the dose is estimated as closely as possible from the information given in the protocols.

P.O.=Administration per os by a dropping pipette or a catheter.

F.=Feeding on bread soaked in a culture of *B. aertrycke*.

E.=Exposure to risk of infection.

Table III.  
*Immunisation against B. aertrycke with dead cultures administered per os.*  
*Tested by intraperitoneal inoculation or per os.*

Series	Method of immunisation			Method of testing			Mortality ratio. Controls: in days: immunised	Average time to death in days: controls
	Living or dead bacteria	Dose in adminis- tration	No. of times adminis- tered	Mortality % from immuni- sation	Route of adminis- tration	Dose in bacilli		
Webster (a) <i>Journ. Exp. Med.</i> xxxvi. 91	D.	F.	28	0	P.O.	10 <sup>6</sup>	75	23
Webster (b) <i>Ibid.</i> xxxvi. 91	D.	F.	28	0	Ip.	10 <sup>6</sup>	100	2-25
Orenstein (a) <i>Zeitsch. f. Hyg.</i> cx. 476	D.	P.O.	4	40	P.O.	10 <sup>10</sup>	100	—

F. = Fed on bread soaked in cultures of *B. aertrycke*.

P.O. = Administered per os with a dropping pipette or with a catheter.

Table IV.  
*Immunisation against B. aertrycke with living cultures administered per os.*  
*Tested by intraperitoneal or subcutaneous inoculation.*

Series	Method of immunisation			Methods of testing			Mortality ratio. Controls: in days: immunised	Average time to death in days: controls
	Living or dead bacteria	Dose in adminis- tration	No. of times adminis- tered	Mortality % from immuni- sation	Route of adminis- tration	Dose in bacilli		
Webster (c) <i>Journ. Exp. Med.</i> xxxvi. 92	L.	F.	5 to 13	28	Ip.	10 <sup>6</sup>	100	1-7
Webster (f) <i>Ibid.</i> xxxvi. 88	L.*	F. + P.O.	30† + 1 P.O.	54	Ip.	2 × 10 <sup>6</sup>	100	—
Webster (h) <i>Ibid.</i> xxxvi. 99	L.	F. + P.O.	34† + 1 P.O.	66-7	Ip.	2 × 10 <sup>6</sup>	100	—
Lange and Yoshioke (e) <i>Zeitsch. f. Hyg.</i> cx. 459	L.	P.O.	10 <sup>6</sup> to 10 <sup>9</sup>	0	Sc.	10 <sup>4</sup>	100	8-3

F. = Feeding on bread soaked in a culture of *B. aertrycke*.

P.O. = Administration per os by a dropping pipette or a catheter.

\* = These mice were fed on an incompletely sterilised vaccine.

† = Number of days over which feeding was continued. This was followed by one administration of living culture per os.

Table V.  
*Immunisation against B. aertrycke with living cultures administered per os. Tested per os.*

Series	Method of immunisation				Method of testing				Mortality ratio, Controls: in days: immunised	Average time to death in days: immunised	Average time to death in days: controls
	Living or dead bacteria	Route of adminis- tration	Dose in bacilli	No. of famies adminis- tered	Mortality % from immuni- sation	Route of adminis- tration	Dose in bacilli	No. of mice tested	Mortality % in controls		
Webster (d) <i>Journ. Exp. Med.</i> XXXIX. 130	L.	P.O.	$4.5 \times 10^8$	1	30	P.O.	$4 \times 10^8$	54	80	2.7	—
Webster (e) <i>Ibid.</i> XXXVI. 88	L.†*	F.	?	30†	50	P.O.	$10^7$	12	58.3	7	—
Webster (g) <i>Ibid.</i> XXXVI. 90	L.	F.	?	34†	61.1	P.O.	$10^7$	7	100	7	—
Lange and Yoshioka (a) <i>Zeitschr. f. Hyg.</i> cr. 457	L.	P.O.	$10^8$ to $10^7$	1	0	P.O.	$10^8$	9	33.3	0.75	14.25
Lange and Yoshioka (b) <i>Ibid.</i> cr. 457	L.	P.O.	$10^8$ to $10^9$	1	0	P.O.	$10^8$	7	83.3	0.97	7.7
Lange and Yoshioka (c) <i>Ibid.</i> cr. 457	L.	P.O.	$10^8$ to $10^7$ + $10^9$	2	40	P.O.	$10^8$	6	83.3	1	7.5
Lange and Yoshioka (d) <i>Ibid.</i> cr. 458	L.	P.O.	$10^7$	1	33.3	P.O.	$10^8$	6	100	1.5	10

\* = These mice were fed on an incompletely sterilised vaccine.

† = Number of days over which feeding was continued.

‡ = The mouse which died showed no definite evidence of *B. aertrycke* infection.

F. = Fed on bread soaked in cultures of *B. aertrycke*.

P.O. = Administered per os with a dropping pipette or with a catheter.

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These 32 mice, therefore, presented a quota of the original population which had been selected in two ways. They formed a sample of a surviving group which amounted in all to half the original population. They formed a unique group, representing 17.3 per cent. of the original population, characterised by the fact that they had survived for one month or more since the last feeding, without ever having been known to excrete *B. aertrycke* in their faeces.

A specimen of blood was obtained from each mouse and tested against type and group suspensions of *B. aertrycke* in a series of dilutions commencing at 1/20. The serum from one mouse gave a trace of agglutination at a dilution of 1/20 with the type suspension, and standard agglutination at 1/160 with the group suspension. The sera from the other 31 mice gave completely negative results.

We decided to test these mice for resistance, by intraperitoneal inoculation, in order to discover whether their apparent escape from infection was associated with an increased resistance to *B. aertrycke* when this organism was introduced directly into the tissues. The first two horizontal lines of Table VI show the result of injecting each of the 32 mice, together with 30 controls, with 200,000 *B. aertrycke* administered intraperitoneally. All mice which died were submitted to post-mortem examination according to the technique which has been recorded in many previous reports.

The results need little comment. During the 21 days which followed the intraperitoneal injection, the total and specific mortality among the controls was 76.7 per cent. Among the selected mice the total mortality was 21.9 per cent., the specific mortality was 12.5 per cent. The average time to death among the controls which succumbed to infection was 7.4 days; among the selected mice which died of *B. aertrycke* infection it was 5 days. The selected mice were apparently about six times as resistant as a normal sample of mice, judged from the specific mortality. They represented a selected sample of about one-sixth of the normal population from which they were originally derived, though their selection on a basis of survival was only in the proportion of one-half.

These selected mice were clearly exceptionally resistant to *B. aertrycke*, when introduced into their tissues, and the results, at this stage, were entirely compatible with the view that their escape from infection during the early stages of the experiment was the result of a natural resistance, which had remained unaltered during this period.

As the result of this first intraperitoneal injection, followed by observation over 21 days, the sample of 32 mice had been subjected to a further sorting process, leaving 25 mice, which formed a sample amounting to 13.5 per cent. of the original 185 mice.

These 25 mice, together with 25 controls, were now injected intraperitoneally with 20,000,000 *B. aertrycke*, and were, thereafter, observed for 68 days. The results are shown in Series C and D of Table VI. All the controls

died within 11 days and all from typical *B. aertrycke* infection. The average time to death was 5.2 days. By the time the last control had died three of the 25 selected mice had succumbed to the same infection. By the 68th day the total mortality among the selected mice was 44 per cent., and the specific mortality 28 per cent. The average time to death among those selected mice which died from *B. aertrycke* infection was 25.6 days. More than half of these selected mice, therefore, withstood for 68 days an infection which killed all the individuals of an equal sample of normal mice within 11 days. Clearly, the previous intraperitoneal inoculation had not acted as a simple selective agent. The mice which survived its effects for 21 days were possessed of a degree of resistance to *B. aertrycke*, when introduced into the tissues, not found among a normal sample. There had been immunisation as well as selection.

Table VI.

*Showing results of successive intraperitoneal inoculations carried out on a selected sample of mice.*

Series	Nature of mice	No. of mice	Bacteria inoculated	Dose administered in bacilli	Days observed	Days to death	Average time to death in days: specific deaths	Total mortality %	Specific mortality %
A	Surviving non-excretors after feeding (see text)	32	<i>B. aertrycke</i>	$2 \times 10^6$	21	3, 4, 6, 7, 8*, 16*, 18*	5	21.9	12.5
B	Normal controls to A	30	<i>B. aertrycke</i>	$2 \times 10^6$	21	1, 2, 2, 3, 3, 3, 4, 4, 5, 6, 6, 8, 8, 8, 9, 9, 10, 11, 11, 11, 13, 15, 18	7.4	76.7	76.7
C	Survivors from A	25	<i>B. aertrycke</i>	$2 \times 10^7$	68	5, 8, 9, 15, 31*, 34, 40, 50*, 54*, 63*, 68	25.6	44	28
D	Normal controls to C	25	<i>B. aertrycke</i>	$2 \times 10^7$	68	2, 2, 3, 3, 3, 3, 3, 3, 3, 4, 4, 4, 4, 4, 5, 6, 6, 6, 7, 8, 8, 9, 9, 11, 11	5.2	100	100
E	Survivors from C	14	<i>Pasteurella</i>	$5 \times 10^8$	28	1, 1, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2	1.8	92.9†	92.9
F	Normal controls to E	20	<i>Pasteurella</i>	$5 \times 10^8$	28	2, 2, 2, 2, 2, 2, 2, 2, 2, 3, 3, 3, 3, 3, 3, 3, 3, 4, 4	2.65	100	100
G	Normal controls to E	3	<i>Pasteurella</i>	$5 \times 10$	28	2, 3, 3	2.7	100	100
H	Normal controls to E	3	<i>Pasteurella</i>	5	28	2	2	33.3	33.3

\*=Death not due to *B. aertrycke* infection.

†=The surviving mouse from this group was re-tested ten days later and found susceptible (see text).

In the studies referred to above, Webster has shown that, among any considerable sample of mice from an inbred strain, a certain proportion will survive infection, and a proportion of these survivors will act as though they were entirely refractory, in the sense that they will neither yield cultures of *B. aertrycke* from their faeces, nor from their blood, nor is it possible to demonstrate agglutinins for that organism in their serum. Using a single strain of *B. aertrycke* this proportion is constant, within moderately narrow limits, for different samples of the same inbred strain, but varies with different strains of mice. Using different strains of *B. aertrycke*, that is, strains which

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would be placed in this group on general cultural and serological grounds, but which have been derived from different sources, and have long been held in stock in different laboratories, and keeping the host factor constant by using a single strain of inbred mice, Webster showed that the result of administration per os differed with the different bacterial strains, as regards the percentage of surviving and refractory mice. These differences became more marked when comparison was made between the action of different bacterial species within the *B. paratyphosus* B-*B. enteritidis* group (Webster, 1923, a, b, c and d).

It seemed probable that the host-resistance displayed in such experiments would prove to be non-specific in character, and Webster proceeded to test this point by submitting mice to the action of a metallic poison, mercuric chloride. He showed (Webster, 1923 d) that by administering suitably graded doses of this substance per os to samples of mice, mortality curves could be obtained similar in their general form to the curves obtained after infection with *B. aertrycke*. In later series of experiments he showed that mice, which had survived one or more doses of mouse typhoid bacilli, were more resistant than normal mice to the action of mercuric chloride (Webster, 1924 a), that, when a strain of mice unusually resistant to *B. aertrycke* infection has been produced by selective breeding, such mice are also unusually resistant to mercuric chloride (Webster, 1924 b), and that mice, whose resistance to *B. aertrycke* infection has been raised by a particular diet, show an increased resistance to this chemical poison. In this last series of experiments it was also shown that the resistant mice were less susceptible than normal mice to the intraperitoneal inoculation of Botulinus toxin (Webster and Pritchett, 1924).

In another series of experiments (Webster, 1924 a), it was demonstrated that the survivors from various groups of mice, to which had been administered different strains of the paratyphoid-enteritidis group of bacteria, showed in all cases an increased resistance to *B. aertrycke* infection, and that the increase in resistance seemed to depend rather on the virulence of the strain used in the primary infection, than on the closeness of the antigenic relationship between the primary infecting strain and *B. aertrycke*.

In the experiment at present under discussion, we had found that a group of mice, selected on the grounds of their apparent failure to react to the ingestion of *B. aertrycke*, were unusually resistant to the intraperitoneal inoculation of this organism, and that, three weeks after this intraperitoneal inoculation, they showed a degree of resistance without parallel among a sample of normal mice. It appeared of interest to test whether these mice were possessed of any increased resistance to an entirely unrelated bacterial infection.

For this purpose we took the 14 survivors from Series C (Table VI), and inoculated them intraperitoneally with 500 *Pasteurella muris*. At the same time we inoculated twenty controls with the same dose, three controls with 50,

and three with 5 *Pasteurella*. We purposely employed a relatively large dose of *Pasteurella*, because the test was, from its nature, one which could not be repeated, and it was necessary to be certain of a high mortality among the controls. In our hands the minimal lethal dose of *Pasteurella* has varied widely, from a single organism to many thousands, when injections are made intraperitoneally into mice.

The results are shown in Series E, F, G and H (Table VI). Of the 14 survivors, 13 died within 2 days. Of the twenty controls which received the same dose, all died within 4 days. Of the three controls which received 1/10th of this dose, all died within 3 days. Of the three controls which received 1/100th of this dose, one died in 2 days and two survived without any evidence of illness. Thus, the 14 survivors from the *B. aertrycke* infections received between 10 and 100 minimal lethal doses of *Pasteurella*. The 13 mice which died showed no delay in the fatal issue. Their average time to death was 1.8 days. The average time to death of the twenty controls which received the same dose was 2.65 days.

The single selected mouse which survived the injection of *Pasteurella* showed no ill-effects whatever. Since it seemed unlikely that one mouse would prove entirely refractory, while its 13 companions seemed more susceptible than the normal controls, the suspicion arose that some error had occurred in the inoculation, and that this mouse had not received the full dose intraperitoneally. After an interval of some 10 days, therefore, this mouse was re-inoculated with the same dose, together with two controls. The selected mouse died in 24 hours, the two controls on the second day.

It would appear that a group of mice, selected by survival after repeated infections with *B. aertrycke* and possessing an enormously increased resistance to that organism, show no increased resistance to *Pasteurella muris*, when compared with a sample of normal mice.

#### LATENT *B. AERTRYCKE* INFECTION AMONG THE SELECTED MICE.

The 14 surviving mice from Series C had been observed for 68 days following the second intraperitoneal injection of *B. aertrycke*. At autopsy, carried out after their death from acute *Pasteurella* infection, a portion of the spleen from each mouse was transferred to a tube of broth and incubated for 24 hours. A copious growth of *Pasteurella* was obtained in each case. From these broth cultures plates of McConkey's lactose-bile-salt medium were inoculated, and any non-lactose fermenting colonies were subcultured and tested by agglutination.

From the spleens of 8 of the 14 mice, cultures of *B. aertrycke* were obtained in this way. It is possible that the abundant growth of *Pasteurella* in the broth cultures hindered the development of *B. aertrycke*, and that, in the absence of this disturbing factor, the proportion of positive results would have been higher. In any case it is demonstrated that the majority of the selected groups were still harbouring *B. aertrycke* in their tissues.

## THE PRESENCE OF AGGLUTININS IN THE BLOOD OF INFECTED MICE.

The rôle of humoral immunity as a factor in survival has been briefly discussed in a recent report (Topley, Ayrton and Lewis, 1924) on the basis of the evidence afforded by the demonstration of agglutinins in the blood of surviving mice.

The relevant data elicited in the course of the present experiment are set out in Table VII.

Table VII.

*Showing results of agglutination tests in the selected group of mice at various dates.*

Record number of mouse*	Time of test					
	Before first intraperitoneal inoculation		14 days after first intraperitoneal inoculation		63 days after second intraperitoneal inoculation	
	Type	Group	Type	Group	Type	Group
No. 47	Trace 20	160	80	320	D.	D.
" 49	—	—	—	640	D.	D.
" 56	—	—	—	40	D.	D.
" 57	—	—	—	40	Trace 20	640
" 63	—	—	—	40	Trace 20	80
" 27	—	—	—	—	Trace 20	40
" 43	—	—	—	—	640	640
" 60	—	—	—	—	320	—
" 62	—	—	—	—	—	160
" 67	—	—	—	—	Trace 20	—
" 80	—	—	—	—	—	80
" 84	—	—	—	—	20	80
No. of mice tested	32		27		16	
No. of mice +	1		5		9	
Percentage of mice +	3.1		18.5		56.25	

\* = This number refers to the record number of the mouse in the original population and has no other significance. Mice which acted negatively throughout are not recorded individually, but are included in the number of mice tested.

D. = Died before this date.

The following facts are clearly shown. Although the selected groups of 32 mice when first tested by intraperitoneal inoculation were about six times as resistant as a sample of normal mice, only one of the 32 mice showed the presence of agglutinins in its serum. Clearly, the presence of agglutinins is no measure of resistance, a fact frequently demonstrated in previous experiments of our own, as also by Webster and by many other workers.

After each intraperitoneal inoculation the percentage of mice showing the presence of agglutinins markedly increases, so that 14 days after the first inoculation 18.5 per cent. of the mice reacted positively, and 63 days after the second inoculation this proportion had been increased to 56.25 per cent. Mice surviving the first intraperitoneal inoculation had acquired a definite increase of resistance as the result of their experience. The mice surviving the second intraperitoneal inoculation were not tested as regards their resistance to *B. aertrycke*, so that we cannot include them in the argument.



It seems clear, however, that if we select mice by submitting them to the action of successive and increasing doses of living *B. aertrycke*, we shall find that increasing resistance is associated with an increasing proportion of positively reacting mice as judged by agglutination.

Only one series of tests in this experiment enables us to make a direct comparison between the fate of mice with agglutinins and mice without, where both groups have passed through the same experience, and both are submitted to the same test inoculation. Unfortunately the figures are very small. On the day of the second series of intraperitoneal tests, the sera of four of the 25 selected mice agglutinated *B. aertrycke* at a dilution of 1/20 or over. The sera of the remaining 21 mice failed to react at this dilution. Of the four agglutinators two died during the next 68 days. One showed the typical lesions of *B. aertrycke* infection, the other showed no such lesions, but *B. aertrycke* was isolated by direct plating from its spleen. Of the 21 non-agglutinators, nine died during this period, six of *B. aertrycke* infection and three from other causes.

There is, then, no evidence that the mice with agglutinins were more resistant than their companions, but the numbers involved are too small to have any real significance.

#### DISCUSSION.

The indications afforded by the experiments recorded in this report, when considered in the light of the results obtained by other workers, appear to be of some interest.

Reviewing the whole of the data together, it is clear that some increase in resistance to *B. aertrycke* may be obtained by the administration of killed cultures of this organism. The level of resistance attained appears never to be very high, and, where the test dose is given parenterally, it is exhibited rather by a delay in the time to death than by an increase in the percentage of survivors, when the mice are observed over a considerable period. When the test dose is given per os, or the treated mice are exposed to the risk of infection, the resistance, as judged by survival, appears to be more effective. The parenteral administration of killed cultures appears to be far more effective in increasing resistance than is their administration per os.

The most striking examples of increased resistance to *B. aertrycke* are, however, to be found among those mice which have survived the administration of living cultures of this organism, whatever route is employed for the test dose. In almost all such cases a certain mortality occurs, following the immunising dose, so that the surviving mice form a selected group, altogether apart from any increase in resistance which may have occurred as the result of the procedure employed for immunisation. There is, moreover, a general parallelism between the severity of the selective process and the degree of resistance exhibited by the survivors, as judged by the percentage mortality and the average time to death following a subsequent dose of living organisms.

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Is the increased resistance of these surviving groups the result of simple selection, or is it the result of some change in the condition of the individual hosts brought about by the previous treatment? It appears to us that there is little doubt that such a change is involved, and is a factor of crucial importance.

We have, in many previous reports, commented on the high proportion of latent infections found among mice which have survived the administration or ingestion of *B. aertrycke*. In a recent paper (Topley and Ayrton, 1924) we tabulated the results of a series of experiments bearing on this point, and we can now add to the data there given further figures, obtained in experiments not yet recorded. The figures at present available are summarised in Table VIII.

Table VIII.

*Showing the proportion of positive spleen cultures obtained P.M. from 431 mice which had survived the administration of B. aertrycke, or exposure to risk of infection.*

Group	History of Group	Number of mice in group	Number of mice with positive spleen cultures	Percentage of mice with positive spleen cultures
1	Survived for 21 days after intraperitoneal or subcutaneous injection of <i>B. aertrycke</i>	50	44	88
2	Survived for 42 days after one feeding with 20,000,000 <i>B. aertrycke</i>	98	60	61.3
3	Survived for 42 days after one feeding with 2,000,000 <i>B. aertrycke</i>	39	13	33.3
4	Survived for 42 days after three or four feedings with 2,000,000 <i>B. aertrycke</i>	59	39	66.1
5	Survivors from seven epidemics of <i>B. aertrycke</i> infection, each lasting three months	185	125	67.6

There seems little doubt that almost all mice, which survive the introduction of living *B. aertrycke* into their tissues, and the majority of those which either survive the per os administration of large or moderate doses, or are submitted to the risk of infection during any considerable epidemic of mouse typhoid, harbour *B. aertrycke* in their spleens over weeks or months, although they may give no evidence of the chronic infection from which they are suffering.

If we consider this fact in the light of the marked superiority, as an immunising agent, of living as contrasted with dead cultures of *B. aertrycke*, we can hardly escape the conclusion that the surviving mice are more resistant because they are suffering from a latent infection. We have, for instance, little doubt that the majority of the original selected group of 32 mice, in the experiment described above, were harbouring *B. aertrycke* in their tissues. It would seem that we are dealing with an example of the "depression" immunity which Morgenroth has described in his studies on superinfection (Morgenroth, 1920).

In this respect, then, our conclusions differ from Webster's. It appears to us most probable that the majority of normal mice, which he regarded as completely refractory to the per os administration of *B. aertrycke*, actually contracted a latent infection, and that to this factor they owed their resistance to the subsequent administration of this, or allied, organisms.

Our results do not, of course, in any way affect Webster's conclusions on the variation of resistance among a normal mouse population, nor do they detract from the fundamental importance of these results. We differ from him only in believing that those mice which are, *ab initio*, more resistant than their fellows, are probably not completely refractory to the administration of *B. aertrycke* in the dose which he employs, but respond by contracting a latent infection, which is compatible with prolonged survival and the entire absence of any evidence of disease, and that such mice are relatively resistant to further infection in consequence of this latent infection, and not merely by virtue of an initial natural resistance, which has allowed them to survive a preliminary selective process, in which their less resistant fellows were eliminated.

As regards the specificity or non-specificity of such immunity, our results clearly should not be regarded as incompatible with those recorded by Webster. He showed that increased resistance to *B. aertrycke* is associated with increased resistance to certain closely allied species of bacteria, to mercuric chloride, and to Botulinus toxin. Our own results would appear to demonstrate that increased resistance to *B. aertrycke* infection is not associated with increased resistance to infection with *Pasteurella*. They would appear to limit the significance of Webster's results, as showing that the high resistance of his surviving mice is not a generalised phenomenon, since it is not effective against all bacterial infections; but the findings are supplementary and not contradictory.

The study of the phenomenon of super-infection has not yet been pushed far enough to yield adequate data as regards the specificity of the reactions concerned. It would seem probable, *a priori*, that the degree of specificity would be less strict than in the case of that acquired resistance, which is associated with, if not dependent on, the appearance of specific antibodies in the blood. We should not, however, expect that a latent infection with a given bacterium would alter the resistance of the host to all other bacterial parasites. Such observations as have been recorded are in accord with these expectations, but the whole problem awaits further investigation. The evidence already available does, however, suggest that this type of resistance is of fundamental importance in the epidemic spread of bacterial infection.

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# ON THE PRESENCE OF PHENOMENA AKIN TO ADSORPTION IN BIOLOGY, AS A SOURCE OF FALLACY IN STATISTICAL INQUIRY.

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(With 2 Diagrams.)

AMONG the formulae which describe relationships found in physics, chemistry and biology is one commonly known as the "adsorption formula." It graduates for a considerable range of experiment the amount of adsorption which takes place at the bounding layers of solids, fluids and gases or immiscible fluids in presence of different concentrations of the substance adsorbed. It may be written in the form

$$x = aC^n$$

where  $x$  is the amount of the substance adsorbed;  $C$  the concentration of the adsorbed substance after equilibrium has been attained;  $n$  is an absolute constant less than unity; and  $a$  is a constant depending in value on the units of measurement. The form of this curve is such that  $x$  increases as  $C$  increases but more and more slowly with each further increase in the value of  $C$ . The formula, though it may arise on the theory of chemical equilibria, is really empirical as it does not hold with high values of the concentration.

The formula was, however, used at a date prior to its application to the problems of adsorption. So far as I can discover it was first introduced by Harcourt and Esson<sup>1</sup> who in the year 1867 used it to graduate the relationship of temperature to the rate of the chemical reaction between  $H_2O_2$  and  $HI$ . In 1875 Dr Farr<sup>1</sup> showed that it closely represented the association of the death-rate and the density of population. In 1897, Pareto<sup>1</sup> recommended it as a graduation formula in the statistics of the distribution of wealth. It was only in 1909 that Freundlich<sup>1</sup> applied it to the theory of adsorption. With the addition of a constant to the abscissa, two subsidiary forms which accurately describe many relationships in biology arise, namely,

$$y = a(c + x)^n, \quad y = a(c - x)^n.$$

It is to be noted that when observations obeying these laws are plotted on double logarithmic paper they lie on a straight line while the value of  $n$  is given by the tangent of the angle between the line through the observations and the abscissa. It is thus easy to determine if the law holds and to evaluate the constant  $n$ .

<sup>1</sup> See References on p. 442.

The range of applications of the formula will now be considered. Three typical instances, chosen out of many others, are illustrated in Diagram I. The first is taken from the domain of economics, but in addition it has an importance in public health. It relates to the distribution of the numbers of houses of different rental in England. It was given and commented on by Mr Goschen (1887) in his Presidential Address to the Royal Statistical Society. Taking the initial rental as that of £10 per annum, the number of houses has been plotted on double logarithmic paper. The ordinates give the number of houses and the abscissae the rentals. The actual observations are denoted by circles; the straight line shown was fitted to these observations by the method of least squares. It is obvious that the formula very accurately describes the data.

As a second example, the distribution of incompetence in the improvident has been chosen. The figures are taken from a report of the Glasgow Parochial Board. The graph shows the comparison between the number of persons (the ordinates) and the number of times (the abscissae) each individual sought admission to the Poor House during a period of six months. Here again it is obvious that the observations are in close relation to the formula. The slope of the line which describes this distribution of incompetence is the same as that which describes the distribution of wealth as given by the rental of houses. This suggests that both depend on the same factors in human nature for which the wealth line describes the distribution of competence and the Poor House line that of its opposite.

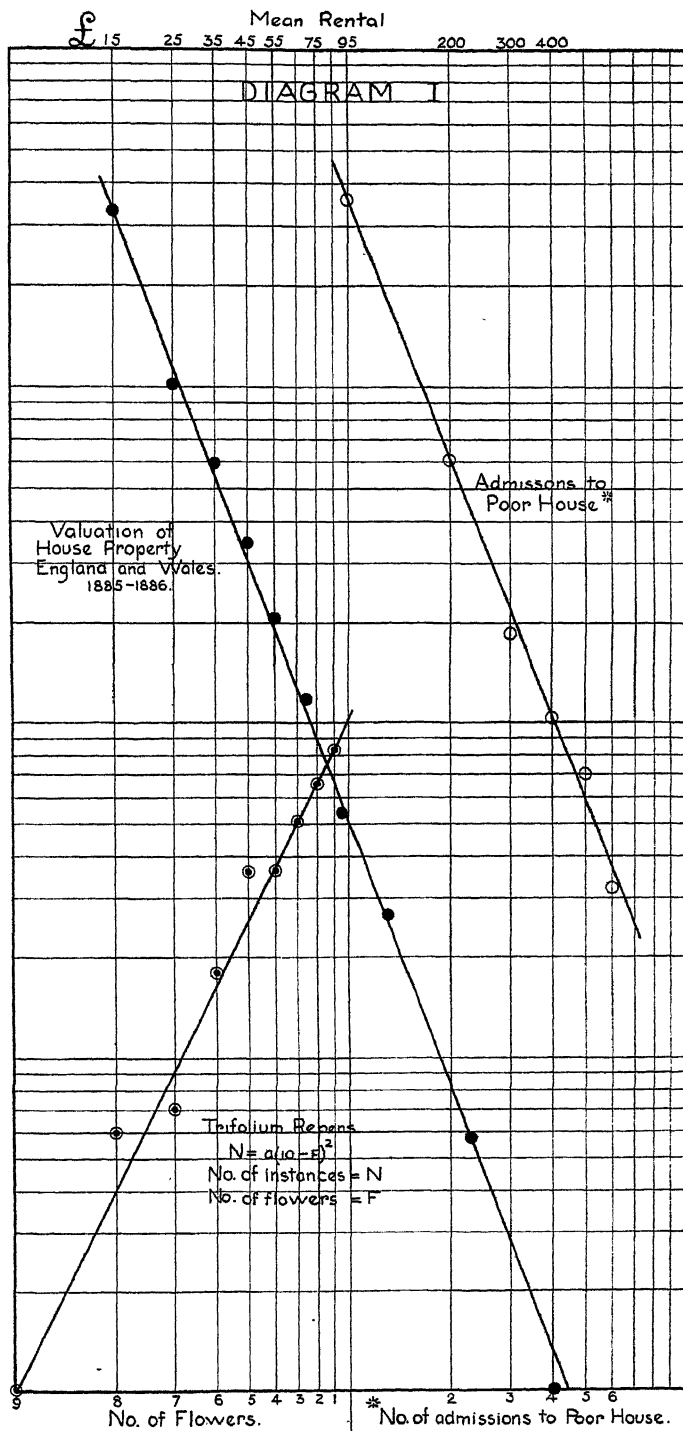
The third example is quoted by Prof. Pearson (1895) from De Vries. De Vries cultivated "a race of *Trifolium repens* in which the axis is frequently prolonged beyond the head of the flower and bears one to ten blossoms." If we look at this from the point of view that each additional blossom requires a greater expenditure of energy and that there is a maximum to the number of blossoms which may result, it is found that taking this maximum as 10 and plotting the frequency against the maximum less the number of flowers found, that the observations lie upon the straight line given by the formula

$$\log N = \log a + 2 \log (10 - F),$$

where  $N$  is the number of instances and  $F$  the total number of flowers.

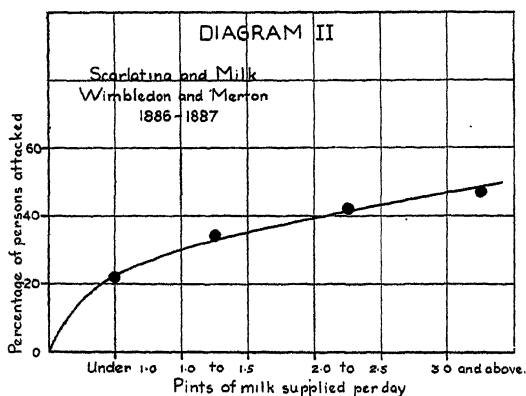
As examples in the realm of biology three have been selected. Dr Ledingham (1912) has applied the formula to the phenomena associated with phagocytosis and finds that the binding of an opsonin and an organism and the phagocytosis of an organism by the leucocytes, both obey the same law, while Dr Martin and Miss Chick (1908) have shown that with certain types of disinfectant, the organism takes up the disinfectant in a manner consistent with the formula and that the destruction of the organism follows the same law.

A third example taken from Carrel and Ebeling (1921) refers to the multiplication of fibroblasts *in vitro* when the growth is stimulated by adding embryonic juice to the medium of cultivation. The increase in the rate of



growth is again closely described by the same type of formula suggesting that the action depends on adsorption.

As has been stated, the formula was first applied in the domain of public health by Dr Farr in 1875, when he used it to describe the relationship of the hygiene of a district to the concentration of the population. In making this application, Dr Farr took the crude death-rate as the measure of the hygiene of the district. The formula was later applied to the standardised death-rate and found not to give a good fit. I have shown elsewhere (Brownlee, 1920), however, that when the life table death-rate is used, the formula holds with a constant index,  $n = 0.1$ , for each decade from 1861-1910. In the Boroughs of London also, the same relationship is found to obtain. The constant  $a$  under the influence of hygienic measures has continually decreased, but the index has not altered.



The formula also describes the data furnished by milk epidemics of scarlet fever (Diagram II). Of these, the most considerable in size was that of Wimbledon for the year 1886-87 (Power, 1886). It was carefully investigated and the observations published in detail. The amount of milk consumed per head in each family per day was recorded and compared with the number of the consumers who developed the disease. It will be observed that the rate of attack increases with the amount of milk consumed, at first rapidly, but the proportion infected to the amount of milk consumed falls with each increase in the amount of milk. The power of infection of the attacking organism varies in a manner consistent with the formula of adsorption. These observations thus confirm the work of Ledingham with reference to opsonins and phagocytosis.

The same phenomenon arises when the death-rates among patients suffering from diphtheria are classified according to the period after the beginning of the illness at which treatment is begun. This is illustrated by figures for Glasgow and London, figures which are in agreement with all others (Table I). It is found that with delay of treatment, the death-rate does not increase without limit. With treatment on the first day, a very low, or no mortality



is found. Treatment on the second day is associated with a larger death-rate, but when treatment is delayed till the fifth or sixth day, a maximum mortality has been attained. Here again something like an adsorption balance between the human organism and the toxin is found. The late use of anti-toxin cannot ensure recovery, though in the event of recovery it may markedly hasten convalescence.

Table I.

*Table showing the percentage mortality when cases of diphtheria are treated on different days of illness.*

Day of treatment	Percentage mortality		
	London, 1895	London, 1896	Glasgow, 1900
1st	4.6	5.3	—
2nd	14.8	14.8	9.0
3rd	26.2	21.9	15.5
4th	33.1	27.8	
5th and after	35.7	31.8	19.8

The phenomena considered have an important bearing on some present day problems in public health. Many inquiries with regard to the effect of environment on health and disease have been carried out and others are in contemplation. A considerable number of these have been conducted in a manner which, to my mind, seriously invalidates the conclusions, the inquiries not extending through a sufficiently wide range of environmental conditions. To illustrate this the facts regarding the density of population and the severity of scarlet fever and enteric fever in Glasgow in the five years 1898–1902 have been chosen. I have selected these figures because Glasgow was divided in 1872 into districts specifically on the ground of their sanitary conditions. The statistics thus afford much more homogeneous data than are obtainable elsewhere. These sanitary districts have been grouped according to the average number of persons per room. In the first column of the table (Table II) the populations of the grouped districts are given; in the second column the number of persons per room; in the succeeding columns the

Table II.

*Table showing the number of cases and mortality of scarlet fever and enteric fever in the sanitary districts of Glasgow, grouped according to room density for the years 1898 to 1902.*

Districts	Population 1901	Room density*	Scarlet fever			Enteric fever		
			Cases	Deaths	Mortality per cent.	Cases	Deaths	Mortality per cent.
Group I	34,868	·5 –1.0	864	20	2.3	106	10	9.4
II	83,255	1.0 –1.5	2148	76	3.5	389	50	12.8
III	201,098	1.5 –2.0	5439	226	4.1	1308	217	15.8
IV	87,885	2.0 –2.25	2184	110	5.0	711	116	16.3
V	237,161	2.25 –2.5	5610	284	5.06	1743	296	16.9
VI	117,445	2.5 –2.75	2091	118	5.6	1003	164	16.3

\* Room density means the average number of persons per room.

number of cases of scarlet fever and enteric fever with their corresponding deaths and death-rates. It will be seen that something resembling the phenomena just discussed occurs here also. Life in unhealthy environment for a certain range of density of population acts so as to depress the vitality but there is a limit to the amount of injury which can take place. This may be different in different diseases. Taking first the statistics of enteric fever, it is seen that had an investigation as to the influence of insanitary conditions on mortality been carried out in those localities alone in which the number of inhabitants per room was over 1.5, the necessary conclusion would be that the environment of the person had no influence on the severity of the disease. The same also applies to scarlet fever but the upper limit of the death-rate is not reached till the concentration of the population is that of two persons per room. It is, however, only because of the very large number of cases of scarlet fever that it is possible to differentiate between the death-rate of Group III, and the death-rates of Groups IV, V and VI. These data regarding scarlet fever have been obtained automatically through the power of compulsory notification. It is not in the least likely that any health inquiry will be able to command the men and money to secure an equivalent number of observations.

I had intended to conclude this note by criticising some recent inquiries which have been carried out without the knowledge that, in addition to statistical fallacies, a biological fallacy might also be present. I have decided, however, to leave the matter as it stands as it is impossible to discuss whether this fallacy affects the conclusions of these statistical papers without working over the original statistical data on which they are based. I content myself therefore, with charting this shoal so that it may be given sea room in future investigations.

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